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The role of metabolism in the anti-tumor cytotoxicity of natural killer cells

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THE ROLE OF METABOLISM IN THE ANTI-TUMOR CYTOTOXICITY OF NATURAL KILLER CELLS

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ABSTRACT

Since their discovery, natural killer cells (NK) cells have been implicated as important players in cancer immunosurveillance. In recent years, researchers have taken advantage of this role by developing NK cell-based immunotherapies in the fight against cancer. While these treatments have been moderately successful against hematological malignancy, they are less effective against solid cancers. This lack of success partially results from the immunosuppressive effects of the tumor microenvironment (TME). While tumors use myriad processes to evade the immune system, the avid consumption of nutrients common to NK and cancer cell metabolism and the production of toxic waste products can have significant deleterious effects on NK cell anti-tumor function.

However, it may be possible to avoid some of this tumor-induced inhibition of NK cell anti-tumor function by manipulating NK cell metabolism and/or environmental conditions. Recent studies have revealed that different activation regimens can affect the metabolic dependencies of different NK cell subsets. Furthermore, studies have identified potential targets in the TME that can make the environment less hostile for infiltrating NK cells. By considering the interrelationship of NK cell metabolism and function—especially in the TME—this thesis illuminates potential strategies to modulate immunometabolic suppression. Despite the promising work already done, many gaps in

the knowledge of NK cell metabolism remain. Future work will need to investigate the specific molecular mechanisms linking metabolism and function, the role of tissue-resident NK cells in cancer immunosurveillance, and the influences of chronic disease and altered systemic metabolism on NK cell anti-tumor activity.

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LIST OF ABBREVIATIONS

γ_c ..... Common cytokine γ-chain

2DG 2-Deoxy-glucose

ACAT2 ..... Acetyl CoA Acetyltransferase 2

ACC ..... Acetyl-CoA Carboxylase

ACLY ..... ATP-citrate lyase

ADCC ..... Antibody-Dependent Cell-mediated Cytotoxicity

AhR ..... Aryl Hydrocarbon Receptor

AMP ..... Adenosine Monophosphate

AMPK ..... AMP-activated Kinase

APC ..... Antigen-Presenting Cell

ARID5B ..... AT-rich Interaction Domain 5B

ATM ..... Ataxia Telangiectasia Mutated protein

ATP ..... Adenosine Triphosphate

BM ..... Bone Marrow

cAMP ..... Cyclic Adenosine Monophosphate

CAR ..... Chimeric Antigen Receptor

CCR ..... Chemokine receptor

CD ..... Cluster of Differentiation

CILP ..... Common Innate Lymphoid Cell Progenitor

CIP4 ..... Cdc42-interacting protein 4

CLP ..... Common Lymphoid Progenitor

cMAC ..... Central Supramolecular Activation Cluster

cNK ..... Conventional Natural Killer

CoA ..... Coenzyme-A

COX ..... Cyclooxygenase

CPT1 ..... Carnitine-palmitoyl Transferase I

CRACC ..... CD2-like Receptor Activating Cytotoxic Cells

Crk ..... CT10 regulator of kinase

CVD ..... Cardiovascular Disease

CXCR ..... CX-chemokine receptor

DAP ..... DNAX-activating protein

DC ..... Dendritic Cell

DNAM-1 ..... DNAX Accessory Molecule 1

EAT-2 ..... Ewing's sarcoma-associated Transcript 2

ECAR ..... Extracellular Acidification Rate

ETC ..... Electron Transport Chain

F-actin ..... Filamentous actin

FAO ..... Fatty Acid Oxidation

FasL ..... Fas Ligand

FASN ..... Fatty Acid Synthase

FasR ..... Fas Receptor

FBP1 ..... Fructose-Bisphosphatase 1

FcγR ..... Crystallizable fragment gamma receptor

FCCP ..... Carbonyl Cyanide-4-(trifluoromethoxy)phenylhydrazone

GAPDH ..... Glyceraldehyde 3-Phosphate Dehydrogenase

GEF ..... Guanine nucleotide Exchange Factor

GG-PP ..... Geranylgeranyl Pyrophosphate

GPCR ..... G-Protein Coupled Receptor

GSK3 ..... Glycogen Synthase Kinase 3

HCMV ..... Human Cytomegalovirus

HEX2 ..... Hexokinase 2

HIF-1α ..... Hypoxia-Inducible Factor 1α

HMGCS1 ..... 3-hydroxy-3-methylglutaryl-CoA synthase 1

HPC ..... Hematopoietic Progenitor Cell

HSC ..... Hematopoietic Stem Cell

ICAM-1 ..... Intercellular Adhesion Molecule 1

ID2 ..... Inhibitor of DNA binding 2

IDO ..... Indoleamine 2,3-dioxygenase

IFN ..... Interferon

IL ..... Interleukin

IL-1RAcP ..... Interleukin-1 Receptor Accessory Protein

ILC ..... Innate Lymphoid Cell

ILC3 ..... Group 3 ILC

ILT-2 ..... Immunoglobulin-Like Transcript 2

IRAK4 ..... IL-1R-associated kinase 4

ITAM.....Immunotyrosine-based Activation Motif

ITIM.....Immunoreceptor Tyrosine-based Inhibition Motif

ITSM.....Immunoreceptor Tyrosine-based Switching Motif

JAK.....Janus Kinase

KIR.....Killer Immunoglobulin Receptor

LAT.....Linker for Activation of T cells

LDHA.....Lactate Dehydrogenase A

LFA-1.....Leukocyte Function-associated Antigen 1

Lin.....Lineage

LMPP.....Lymphoid-Primed Multipotential Progenitor

LN.....Lymph Node

MALT.....Mucosa-Associated Lymphoid Tissue

MAPK.....Mitogen-Activated Protein Kinase

MCMV.....Murine Cytomegalovirus

MCT.....Monocarboxylate Transporter

MHC.....Major Histocompatibility Complex

MIC.....MHC class I chain-related protein

MIF.....Macrophage Migratory Inhibitory Factor

MIP.....Macrophage Inflammatory Protein

miR.....MicroRNA

MTCO2.....Mitochondrial Cytochrome C Oxidase

MTOC.....Microtubule-organizing Center

mTOR.....Mechanistic Target of Rapamycin

mTORC ..... mTOR complex

MyD88 ..... Myeloid Differentiation primary response protein 88

NAD+ ..... Nicotinamide Adenine dinucleotide

NADH .....NAD and hydrogen

NB .....Neuroblastoma

NCAM ..... Neural Cell Adhesion Molecule

NCR.....Natural Cytotoxicity Receptor

NF-κB..... Nuclear Factor kappa-light-chain-enhancer of activated B cells

NFAT .....Nuclear Factor of Activated T Cells

NK .....Natural Killer

NKDI..... Natural Killer Cell Developmental Intermediate

NKG2 ..... Natural Killer Group 2

NKP..... Natural Killer Cell Progenitor

NKp .....Natural Killer protein

NTAL ..... Non-T cell Activation Linker

OCR..... Oxygen Consumption Rate

OXPHOS .....Oxidative Phosphorylation

PB..... Peripheral Blood

PBMC..... Peripheral Blood Mononuclear Cell

PGE2 ..... Prostaglandin E2

PI3K ..... Phosphoinositide 3 Kinase

PKM1 ..... Pyruvate Kinase Isozyme 1

PLC ..... Phospholipase C

pMAC ..... Peripheral Supramolecular Activation Cluster

Poly (I:C) ..... Polyinosinic-polycytidylic acid

PPAR ..... Peroxisome Proliferator-Activated Receptor

PPI ..... Proton Pump Inhibitor

PSGL-1 ..... P-selectin glycoprotein ligand 1

RAG1 ..... Recombination Activating Gene 1

RANTES ..... Regulation on Activation, Normal T cell Expressed and Secreted

RIG-I ..... Retinoic acid-inducible Gene I

RORγt ..... Retinoic-acid-related orphan nuclear receptor γ

S6K ..... S6 ribosomal kinase

SAP ..... SLAM-associated protein

SASP ..... Senescence-Associated Secretory Phenotype

SCD1 ..... Stearoyl-CoA Desaturase 1

SDHB ..... Succinate Dehydrogenase

SH2 ..... Src-homology domain 2

SHIP-1 ..... SH2 domain-containing Inositol 5' Phosphatase 1

SLAM ..... Signaling Lymphocytic Activation Molecule

SLT ..... Secondary Lymphoid Tissues

SNARE ..... Soluble *N*-ethylmaleimide-sensitive factor Attachment protein Receptor

SREBP ..... Sterol Regulatory Element Binding Protein

SRIR ..... Self-recognizing inhibitory receptor

STAT ..... Signal Transducer and Activator of Transcription

TAM ..... Tumor-associated Macrophage

TCA ..... Tricarboxylic Acid Cycle

TCR ..... T cell Receptor complex

tdNK ..... Tissue-Derived NK Cell

TGF-β ..... Transforming Growth Factor β

Th ..... T helper cell

TLR3 ..... Toll-like Receptor 3

TME ..... Tumor Microenvironment

TNF ..... Tumor Necrosis Factor

TOFA ..... 5-(tetradecyloxy)-2-furoic acid

TRAF6 ..... TNFR-associated factor 6

TRAIL ..... TNF-related Apoptosis-inducing Ligand

Treg ..... Regulatory CD4+ T Cell

trNK ..... Tissue-resident Natural Killer

TYK2 ..... Tyrosine Kinase 2

ULBP(1-6) ..... UL16-binding protein

UQCRB ..... Ubiquinone-cytochrome c Reductase Binding protein

VAMP7 ..... Vesicle-Associated Membrane Protein 7

VEGF ..... Vascular Endothelial Growth Factor

WASp ..... Wiskitt-Aldrich Syndrome protein

ZAP70 .....Zeta chain of TCR Associated with Protein 70

INTRODUCTION

Cancer immunosurveillance—the idea that the immune system suppresses nascent tumor growth—was proposed in the first decade of the 1900s. Yet it took nearly a century to prove it (Ribatti, 2016; Vesely, Kershaw, Schreiber, & Smyth, 2011). Through a series of human epidemiological studies and murine experiments, researchers demonstrated that not only does the immune system eliminate tumors, it sculpts the tumors that ultimately "escape" in a process now called "immunoediting" (Dunn, Bruce, Ikeda, Old, & Schreiber, 2002). This begs the question: what if instead of suppressing the immune system, (as standard treatments often do) therapies unleashed the immune system? Here enters immunotherapy. As a technique that either enhances a patient's endogenous immune system or "adoptively" transfers immune cells to him or her, immunotherapy is fast becoming an important treatment modality for myriad cancer types (Mellman, Coukos, & Dranoff, 2011; Vanneman & Dranoff, 2012; Wayteck, Breckpot, Demeester, De Smedt, & Raemdonck, 2014). While standard treatments such as chemotherapy, radiation, and surgery may be effective for primary tumors, curing metastatic disease remains elusive (Wayteck et al., 2014). Given the potential geographic ubiquity of immune cells, immunotherapy may very well be the answer to metastatic malignancy. However, the results have been mixed. Though immunotherapy has been very effective for hematological cancers, the data on solid tumors is less promising (Fang, Xiao, & Tian, 2017). In the past few years, as the scientific community has probed deeper into this

dichotomy, it has begun to reexamine one of the most basic requirements for cell function—metabolism (E. J. Pearce & Pearce, 2017).

Increasingly, it is becoming clear that not only does metabolism drive function, function drives metabolism (Hotamisligil, 2017; Poznanski, Barra, Ashkar, & Schertzer, 2018). Often, immune cells that have infiltrated the tumor microenvironment (TME) exhibit deranged metabolic phenotypes and poor function (Beckermann, Dudzinski, & Rathmell, 2017; Mohamed, Al-Khami, & Rodriguez, 2018; Sugiura & Rathmell, 2018). What if the metabolism of immune cells could be manipulated? What if immune cells could be rendered impervious to the conditions of a hostile tumor milieu that frequently has poorly developed vasculature, depleted nutrients, and high concentrations of toxic metabolites (Chang et al., 2015; Prado-García & Sánchez-García, 2017; Singer, Cheng, Kreutz, Ho, & Siska, 2018; Wilson & Hay, 2011)? This could be the future of immunotherapy. But first, it is important to explore the complex interplay between immune function and metabolism, so-called "immunometabolism." Much of immunometabolic research has focused on T-cells and macrophages (P. J. Murray, Rathmell, & Pearce, 2015; O'Neill, Kishton, & Rathmell, 2016; E. L. Pearce & Pearce, 2013). In these studies, it has been shown that each immune subset exhibits particular metabolic phenotypes that support their unique role in the immune ecosystem (Klein Geltink, Kyle, & Pearce, 2018; Van den Bossche, O'Neill, & Menon, 2017). However, one immune cell type known to be important to cancer immunosurveillance is conspicuously absent in these early studies—the natural killer (NK) cell (Gardiner & Finlay, 2017; Malmberg et al., 2017). And while there has been growing interest in the

immunometabolism of NK cells over the past few years, there has been limited review on the role of metabolism in NK cell anti-tumor activity (Gardiner, 2019). This thesis will provide a more comprehensive analysis. First, this thesis will introduce NK cells, their normal function, and their role in cancer immunosurveillance. Second, this thesis will review several major metabolic pathways and explicitly consider their potential influences on NK cell function. Third, this thesis will survey the effects of the TME on NK cell metabolism and function. At its conclusion, the author hopes that readers will understand the fundamental relationship between metabolism and function in NK cells and be familiar with possible strategies to use that relationship to enhance NK cell anti-tumor activity.

FOUNDATIONS OF NATURAL KILLER CELL BIOLOGY

In the 1960s, the NK cell didn't exist. It was artifact—the "normal reactivity" against tumor cell lines to be either controlled for or ambiguously explained away (Hellström, Hellström, Pierce, & Bill, 1968; Oldham, 1983; Rosenau & Moon, 1961; Smith, 1966). That all changed in the 1970s when researchers began to investigate this "artifact," exploring the "natural cytotoxic reactivity of mouse lymphoid cells." The "natural killer" cell was born (Herberman, Nunn, Holden, & Lavrin, 1975; Herberman, Nunn, & Lavrin, 1975; Kiessling, Klein, Pross, & Wigzell, 1975; Kiessling, Klein, & Wigzell, 1975). Initially, NK cells were only characterized as large granular lymphocytes that could "naturally" (i.e. without prior sensitization) kill virally-infected or malignantly transformed cells. However, researchers have since discovered that NK cells also modulate immune responses through the secretion of cytokines and chemokines, which affect recruitment and function of various immune cells (e.g. T cells, neutrophils, and dendritic cells (DC)) (M. A. Cooper, Fehniger, Turner, et al., 2001; Fauriat, Long, Ljunggren, & Bryceson, 2010; Schuster, Hurrell, & Tacchini-Cottier, 2013; Vivier, Tomasello, Baratin, Walzer, & Ugolini, 2008; Waggoner, Cornberg, Selin, & Welsh, 2012). NK cells share this second function with a broader category of immune cells, known as innate lymphoid cells (ILCs). ILCs exhibit diverse roles in immunity and tissue repair, but all produce cytokines, rely on the transcriptional repressor inhibitor of DNA binding 2 (ID2) and require the common cytokine receptor γ-chain (γ_c; also known as interleukin (IL)-2Rγ). Furthermore, researchers divide ILCs into three major divisions—groups 1, 2, and 3—based on cytokine production. NK cells are classified as the

prototypical member of group 1 ILCs, because they produce type 1 cytokines such as interferon-γ (IFN-γ) yet cannot produce either type 2 (e.g. IL-4, IL-5, IL-13) or type 3 cytokines (e.g. IL-17, IL-22). Similarly, group 2 ILCs secrete type 2 cytokines while group 3 ILCs secrete type 3 cytokines (Spits et al., 2013). Note that these cytokine classifications match CD4+ T helper (Th) cell-associated cytokines wherein Th1, Th2, and Th17 produce the same cytokines as ILC groups 1, 2, and 3, respectively (Scoville, Freud, & Caligiuri, 2017; J. Zhu, Yamane, & Paul, 2010).

Though ILCs (including NK cells) have many functional and phenotypic features in common with T cells, ILCs do not express several markers specific for other leukocytes. Hence, one can distinguish ILCs by the lack of specifying "lineage" (Lin) markers expressed on T cells (e.g. CD3, CD5), B cells (e.g. CD19, CD20), and myelomonocytic cells (e.g. CD13, CD14) (Freud, Mundy-Bosse, Yu, & Caligiuri, 2017; Scoville et al., 2017). Moreover, NK cells are immunophenotypically (e.g. by flow cytometry) distinguished as CD45+ lymphocytes which express CD16 (FcγRIIIA) and CD56 (a neural cell adhesion molecule (NCAM)) (L. L. Lanier, Le, Civin, Loken, & Phillips, 1986). Sometimes the surface expressions of markers like CD94/NKG2 heterodimers, natural killer protein 80 (NKp80) and killer immunoglobin-like receptors (KIRs) are also included in characterizing NK cells (Moretta et al., 2014). Several of these surface markers will be discussed in greater detail later.

The differential expression of one of these markers—CD56—has traditionally been used to divide mature peripheral blood (PB) NK cells into two major subsets—$CD56^{bright}$ and $CD56^{dim}$. These two subsets will be revisited later, but for now it is worth

revealing that they have significant functional and phenotypic differences. In general, $CD56^{bright}$ NK cells exhibit greater cytotoxicity, are $CD16^{hi}$, and comprise the vast majority (>90%) of PB NK cells, while $CD56^{dim}$ NK cells more readily produce cytokines (including IFN-γ), are $CD16^{lo/-}$, and preferentially localize to secondary lymphoid tissues (SLTs) such as lymph nodes (LN) (M. A. Cooper, Fehniger, & Caligiuri, 2001; L. L. Lanier et al., 1986). One could imagine how clinical applications of NK cells might require the enrichment of one or both of these subsets, depending on the circumstances. Even more, the functional diversity of NK cells extends far beyond this simple dichotomy. (Peng & Tian, 2017; Sojka, Tian, & Yokoyama, 2014). To better understand this heterogeneity, this section will detail how NK cells develop, how they work, and where to find them.

A. NK Cell Development and Maturation

1. Model Overview

Comprising 5-20% of peripheral blood mononuclear cells (PBMCs), NK cells are one of three major lymphocyte lineages, which include B cells, T cells, and ILCs. (Freud et al., 2017; Langers, Renoux, Thiry, Delvenne, & Jacobs, 2012). Like B and T cells, NK cells derive from the multipotent CD34+ hematopoietic stem cell (HSC). Most leukocytes (excluding T cells) develop exclusively within the bone marrow (BM), and for decades this was thought to be true of NK cells as well (Blom & Spits, 2006; Colucci, Caligiuri, & Di Santo, 2003; Kondo, Scherer, King, Manz, & Weissman, 2001). This

view was supported by early studies in which researchers generated NK cells from the culture of human C34+ HSC in either BM-derived stroma or with IL-15 supplementation (which can be produced by BM stromal cells) (Miller, Alley, & McGlave, 1994; Mrózek, Anderson, & Caligiuri, 1996). However, recent *ex vivo* characterization of CD34+ NK progenitor cells and putative NK cell developmental intermediates (NKDI) has indicated that the latter tend to be enriched in extramedullary tissues such as SLTs (often in residence with unique, mature NK cells subsets), implying that NK cells may develop in these peripheral tissues (Figure 1) (Eissens et al., 2012; Freud et al., 2005; Scoville et al., 2017).

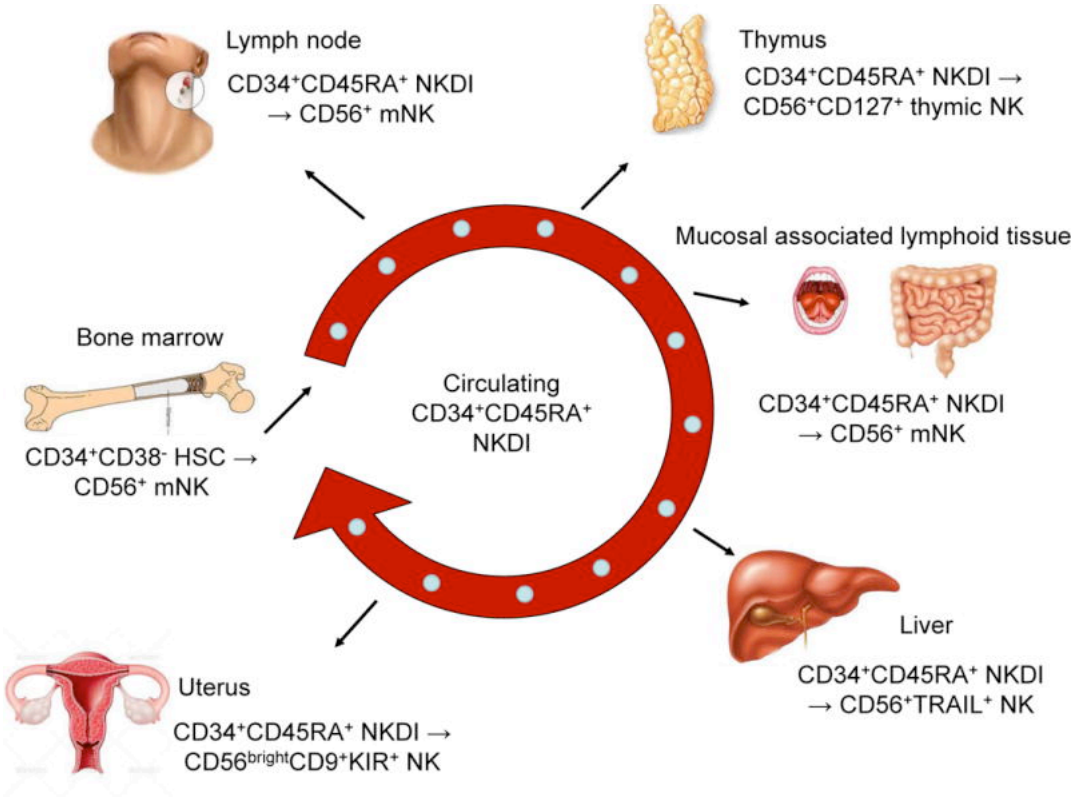

Figure 1. Extramedullary Human NK Cell Development Generates Tissue-Specific Phenotypes. CD34+/CD45RA+ cells are NK developmental intermediates (NKDI) that likely originate in bone marrow (BM). While this subset constitutes <1% of CD34+ progenitors (including hematopoietic stem cells (HSCs)/hematopoietic progenitor cells (HPCs) in BM, it is enriched at 5-10% in blood and >90% of CD34+ progenitor cells reside in secondary lymphoid tissues (SLTs). HPCs likely exit the BM and differentiate into functionally and phenotypically distinct NK cell subsets. Adapted from (Yu et al., 2013).

Over a decade ago, researchers described five stages of human NK cell development, distinguishing them according to expression of CD34, CD117, CD94, and CD16 (Freud & Caligiuri, 2006; Freud et al., 2006; Grzywacz et al., 2006).

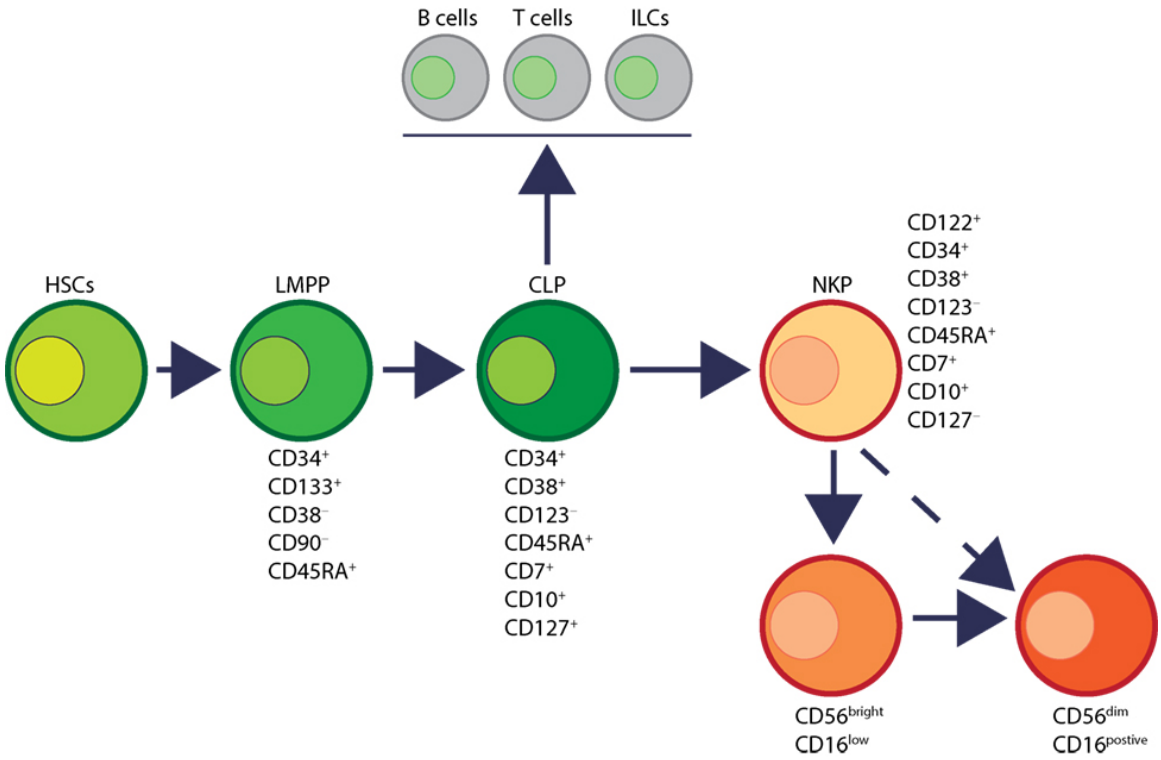

Figure 2. Five-stage Model of Human NK Cell Development. Lin-/CD34+ HSCs differentiate into CD45RA+ lymphoid-primed multipotential progenitors (LMPPs). LMPPs transition to common lymphoid progenitors (CLPs) with expression of CD38, CD10, CD7 and CD127. CD122 expression identifies NK progenitors (NKPs) while CD56 appears in mature NK cells. Adapted from (Abel, Yang, Thakar, & Malarkannan, 2018)

The classic five-stage model starts with a Lin-/CD34+/CD117-/CD94-/CD16- HSC, which differentiates into a Lin-/CD34+/CD117+/CD94-/CD16-/CD45RA+ lymphoid-primed multipotential progenitor (LMPP) in Stage 1 (Figure 2). With expression of CD38, CD10, CD7, and CD127, LMPP becomes a common lymphoid progenitor cell (CLP) (Abel, Yang, Thakar, & Malarkannan, 2018; Luetke-Eversloh, Killig, & Romagnani, 2013; Scoville et al., 2017). CLPs are able to commit to differentiate to Pro-B, Pre-T, natural killer cell progenitor (NKP) cells, or ILCs (Renoux

et al., 2015). When CLPs express CD122 (IL-2/15Rβ), this represents a commitment to the NK cell lineage. In fact, with exogenous administration of IL-15 and media *in vitro,* one can induce such cells to develop into functionally mature NK cells (Freud et al., 2006). Expression of CD56 in later stages identifies an NK cell as mature, with $CD56^{bright}$ NK cells often thought to mature into $CD56^{dim}$ NK cells. More recent models sometimes include a sixth stage wherein $CD56^{dim}$ NK cells begin to express CD57. Meanwhile, further classification of adaptive or "memory-like" NK cells is defined by increased NKG2C expression. It is not clear if one of both CD56 NK subsets can differentiate in these "memory" cells (Abel et al., 2018; Kared et al., 2018; Min-Oo, Kamimura, Hendricks, Nabekura, & Lanier, 2013; Wagner & Fehniger, 2016; Jianhua Yu, Freud, & Caligiuri, 2013).

While the above model presents a good foundation, further investigation has revealed these stages are more heterogeneous than previously realized. For instance, a few years ago researchers discovered that two distinct subsets of stage 2 could be defined by the presence of IL-1R1 (IL-1β receptor) (Scoville et al., 2016). IL-1R1- (stage 2a) and IL-1R1+(stage 2b) NKDI both express the previously identified stage 2 markers, but stage 2b expresses CD161—a pan-ILC surface antigen. Moreover, stage 2b cells lack detectable recombination activating gene 1 (*RAG1*) mRNA (critical to B and T cell development), which is present in both stage 1 and stage 2a cells. In series of experiments in immunodeficient mice, it was shown that while stage 2b cells were restricted to ILC development, stages 1 and 2a retained T cell and DC developmental potential (Scoville et

al., 2016). Hence, CLP might be better classified as stage 2a while stage 2b is classified as a common ILC progenitor (CILP) in humans (Figure 3).

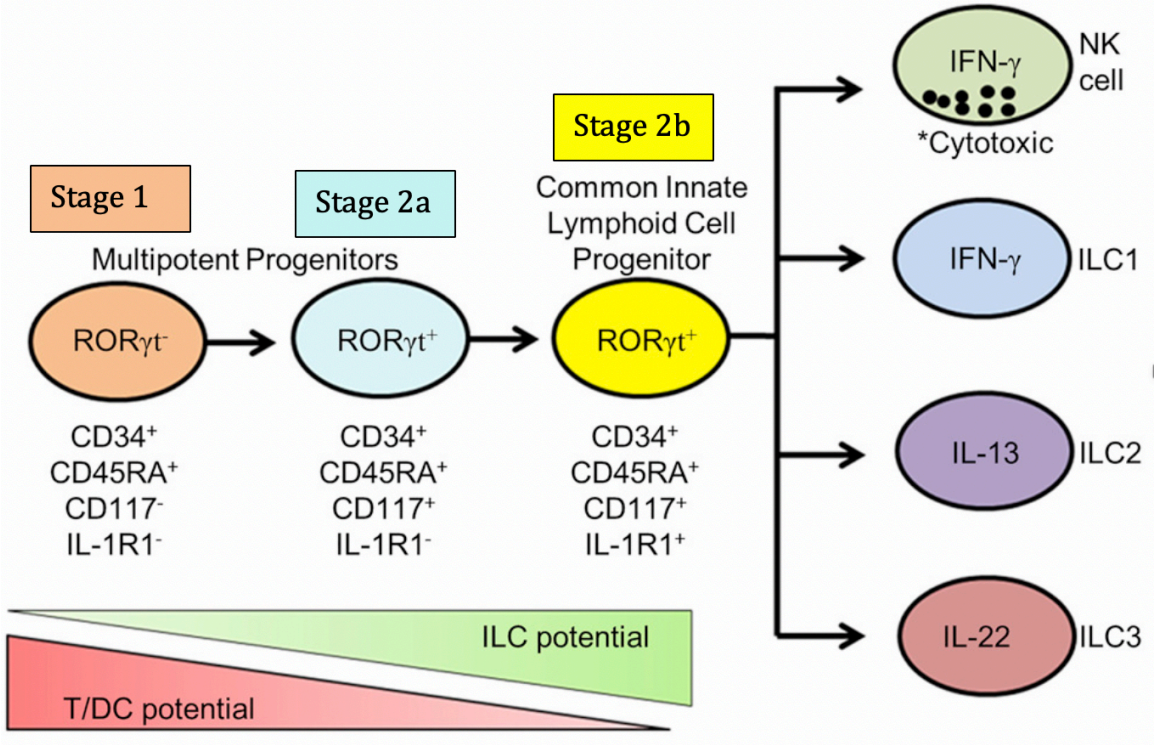

Figure 3. Stage 2 in NK Cell Development Consists of Two Distinct Subsets. Stage 1/LMPP and stage 2a (IL-1R1-) in model of NK cell development have potential to develop into ILCs, T cells, and DCs while stage 2b (IL-1R1+) maturation is restricted to ILC lineage. Note: Retinoic-acid-related orphan nuclear receptor γ (RORγt) is a transcription factor expressed in all ILCs, including PB NK cells. Adapted from (Scoville et al., 2016).

In the previously described model, stage 3 roughly corresponded with NKP (Figure 2). However, given that stage 3 cells are known to secrete IL-22, this would technically classify them as group 3 ILCs (ILC3) (Marina Cella et al., 2009; Cupedo et al., 2009; Hughes et al., 2009, p. 3; Spits et al., 2013). Consequently, whether or not ILC3s and stage 3 NKDI are distinct cell types remains an open question. Regardless,

stage 3/ILC3s have been shown to exhibit significant heterogeneity in expression of myriad surface antigens such as CD62L, CD7, CD56, and NKp44 (Björklund et al., 2016; Cupedo et al., 2009; Freud et al., 2006; Hoorweg et al., 2012).

In stage 4, there exists another bifurcation according to expression of an activating C-type lectin receptor, NKp80. Stage 4a is NKp80-, while stage 4b is NKp80+. Stage 4b proves capable of IFN-γ production or the perforin-dependent cytotoxicity (discussed later) found in mature NK cells. In contrast, stage 4a displays more phenotypic similarities (e.g. IL-22 expression) with stage 3/ILC3s, which confirms its status as an intermediate between NKDI of stages 3 and 4b (Figure 4) (Freud et al., 2016; Scoville et al., 2017).

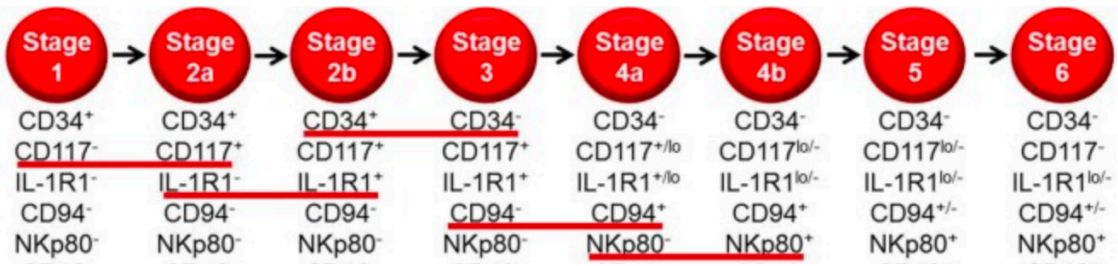

Figure 4. Modern Model of Human NK Cell Development in SLTs. Updated schematic of NK cell development comprising six stages. Red lines underline defining changes in surface antigen expression. Differential expression of CD34, CD117, IL-1R1, CD94, and NKp80 define each stage. Adapted from (Scoville et al. ,2017).

While the described model might very well account for development of "conventional" NK (cNK) cells, it seems inadequate to capture the broad diversity of tissue-resident NK (trNK) cells (i.e. those found in peripheral tissues such as the liver,

gut, and uterus (**Error! Not a valid bookmark self-reference.**)) (Lugthart et al., 2016; Peng & Tian, 2017; Sojka, Plougastel-Douglas, et al., 2014). For example, Renoux et al recently characterized a NK cell lineage-restricted progenitor found in blood, BM, and SLTs. The described NKP was Lin-/CD34+/CD38+/CD123-/CD45RA+/CD7+/CD127- but only gave rise to NK cells. Furthermore, the progenitor lacked the surface markers (e.g. CD161, CD117, CD127) of stage 2b (CILP) (Renoux et al., 2015). Consequently, the modern model of human NK cell development in SLTs fails to capture this progenitor's stage, because it shares the immunophenotype of stage 1 and 2a cells and the developmental potential of stage 3/4a.

In the future, it is likely that models for NK cell development will become increasingly complex as researchers integrate new findings into the ontogeny of ILCs and distinct trNK cell subsets. Hopefully, that corresponds with greater understanding of ways in which to guide NK cell development to improve clinical outcomes where NK function is paramount to disease management. In the next section, this thesis will look more closely at mature human NK cell subsets, including cNK and trNK cells.

2. Mature NK Cell Subpopulations

Briefly, this thesis discussed the two major cNK cell subsets—$CD56^{bright}/CD16^{lo/-}$ and $CD56^{dim}/CD16^{hi}$. As their name implies, traditionally these subsets have been identified based on differential expression of CD56 (NCAM-1) and CD16, a receptor important in antibody-dependent cell-mediated cytotoxicity (ADCC, discussed later) (L. L. Lanier et al., 1986; L. L. Lanier, Le, Phillips, Warner, & Babcock, 1983) Recall that

these subsets, though first studied in PB, are known to have different localization and functional properties. These properties can be understood in term of the surface marker or intracellular protein expression. While cNK cells subsets share many expression profiles for proteins such as the common IL-2 and IL-15 receptor β (IL-2/15Rβ, CD122), 2B4, DNAX accessory molecule 1(DNAM-1), NKG2D, NKp30, and NKp80 (activation/inhibition receptors, see in next subsection), there are a variety of surface markers that cNK subsets do not share (Michael A. Caligiuri, 2008; Freud et al., 2017; Lewis L. Lanier, 1998; Moretta et al., 2014). For example, $CD56^{bright}$ cells have increased expression of CD94/NKG2A, NKp46, IL-2Rα (CD25, high affinity IL-2 receptor), and IL-7Rα (CD127) (M. A. Caligiuri et al., 1990; Michael A. Caligiuri, 2008; Romagnani et al., 2007). One could imagine that expression the last two receptor subunits might allow $CD56^{bright}$ NK cells to be selectively influence by IL-2 or IL-7. In contrast, $CD56^{dim}$ NK cells more avidly express CD16, granzymes, and perforin (proteins associated with cell-mediated cytotoxicity). This subset also exhibits greater expression of CD94/NKG2C and KIRs (Table 1) (M. A. Cooper, Fehniger, & Caligiuri, 2001).

All of this may partially explain the functional dichotomy addressed earlier, in which $CD56^{dim}$ NK cells have greater direct cytotoxicity when engaged with target cells while $CD56^{bright}$ NK cells more readily produce cytokines when stimulated with monokines (i.e. IL-2, IL-12, IL-15, IL-18, and IL-1β) secreted by monocytes, DCs, and T cells (Megan A. Cooper, Fehniger, Ponnappan, et al., 2001; Fehniger et al., 1999). However, it should be noted $CD56^{bright}$ NK cells may display remarkable cell-mediated cytotoxicity with *in vitro* stimulation and $CD56^{dim}$ NK cells can rapidly secrete abundant

cytokines with target cell contact (Björkström et al., 2010; Michel et al., 2016; Strauss-Albee et al., 2015). This demonstrates a now recurring theme: NK cell function shows more complexity than previously imagined.

Table 1. Differential Expression of Selected Molecules in cNK cells. Expression Key: b = high density; c = low density; d = variable; e = majority of cells; f = none; g = upregulated when activated. Adapted from (Cooper et al., 2001a).

	$CD56^{bright}$	$CD56^{dim}$
CD56	++[b]	(+)[c]
CD16	−/+[d]	++
NK receptors		
KIR	−/+	++
CD94	++	−/+
NKG2A	+[e]	−/+
ILT-2	−[f]	+
Cytokine and chemokine receptors		
IL-2Rαβγ	+	−
IL-2Rβγ	+	+
c-kit	+	−
IL-1RAcP	+	+
IL-1RI	+	−/+
IL-18R	+[g]	−/+
CCR7	++	−
CXCR3	+	−/+
CXCR1	−	++
CX_3CR1	−	++
Adhesion molecules		
CD2	++	+
L-selectin (CD62L)	++	−/+
PEN5−PSGL-1	−	+
LFA-1	(+)	++
CD44	++	+
CD49e	++	+

Nonetheless, it may be appropriate to conclude that the two major subsets of cNK cells represent specialization in each of NK cell major functions: cell-mediated cytotoxicity and immunomodulation via cytokine production (Freud et al., 2017). In fact, the preponderant localization of $CD56^{bright}$ NK cells to LNs (alluded to earlier) supports this view, given that these cells often reside in parafollicular regions near T cell and DCs where immune cells can modulate the activity of each other (Megan A. Cooper, Fehniger, Fuchs, Colonna, & Caligiuri, 2004; Fehniger et al., 2003; Ferlazzo, Pack, et al., 2004; Ferlazzo, Thomas, et al., 2004).

$CD56^{bright}$ NK cells predominate not only in LNs, but also in visceral adipose tissue, the kidneys, the uterus, the liver, and mucosa-associated lymphoid tissue (MALTs, of gastrointestinal mucosa) (Carrega et al., 2014). Meanwhile, historically $CD56^{dim}$ reside in PB in higher proportions, yet they also predominate in BM, lung, subcutaneous fat, breast tissue, and spleen (Freud et al., 2006). Consistent with these different localization patterns, the two major cNK cell subsets demonstrate different chemokine receptor expression profiles. For instance, $CD56^{bright}$ NK cells uniquely express chemokine receptors and proteins that facilitate homing to SLTs or other peripheral tissues such as CX-chemokine receptor 3 (CXCR3), chemokine receptor 7 (CCR7), and CD62-L (L-selectin). By comparison, $CD56^{dim}$ NK cells seldomly express these receptors but uniquely express sphingosine-1-phosphate receptor 5 (S1P5), CXCR1, CXCR2, and CX3CR1, which direct this subset away from LN and towards PB (Carrega et al., 2014; Cichocki et al., 2016; Maghazachi, 2010).

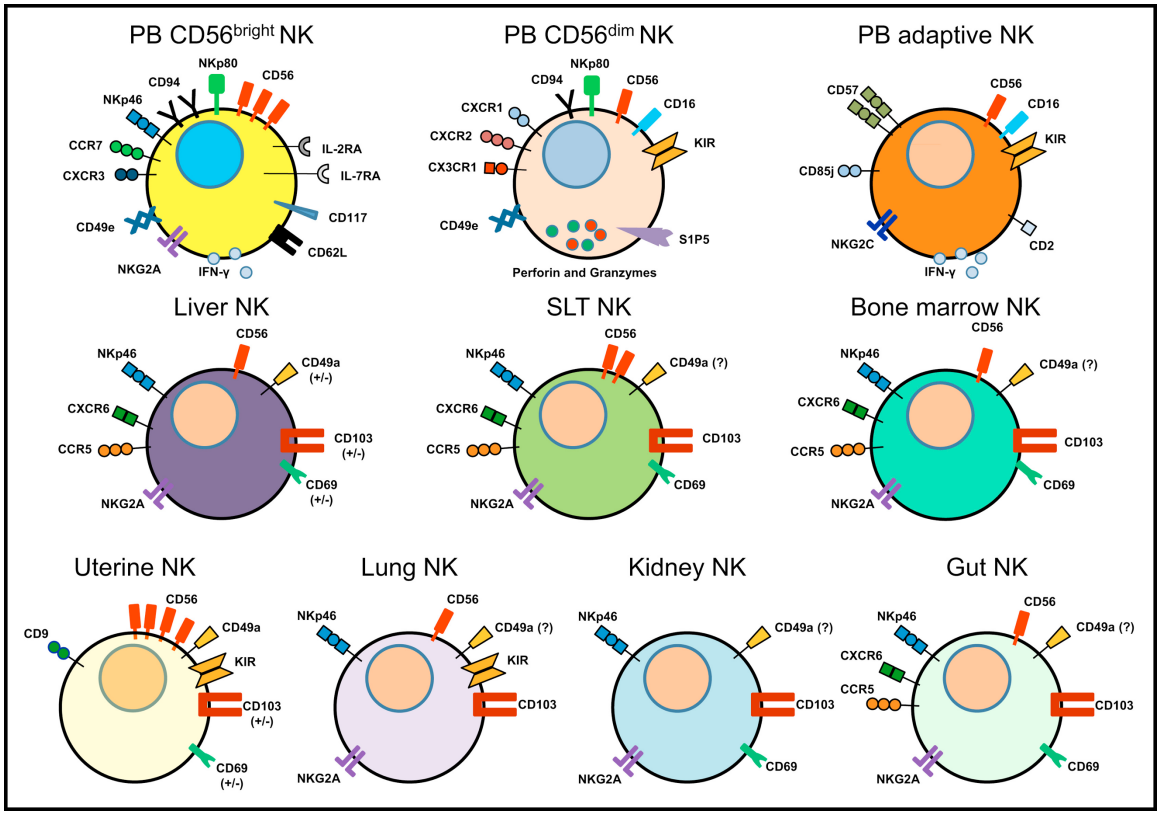

Figure 5. Human NK Cell Diversity: Immunophenotypes of cNK and trNK Cells. Given the incredible phenotypic diversity of NK cells, it is more appropriate to consider distinct subsets identified according to tissue residence rather than traditional CD56^{bright} vs. CD56^{dim} designation. Note that for each surface marker, number of pictograms depicts relative level of expression on associated cell type. Adapted from (Freud et al., 2017).

A closer look at the CD56^{bright} NK cells occupying tissues in recent years has revealed that they differ substantially from those of circulating (PB) CD56^{bright} NK cells. As an example, CD56^{bright} NK cells in tonsils and LN display bimodal expression of DNAM-1, increased relative expression of CD54 (Intercellular adhesion molecule 1 (ICAM-1)), and decreased relative expression of CD56 compared with PB CD56^{bright} NK cells (Lugthart et al., 2016; Lünemann, Vanoaica, Azzi, Nadal, & Münz, 2013). Meanwhile, uterine NK cells have relatively increased expression of CD56, CD9, and

KIRs. Furthermore, uterine cells express splice variants for genes that encode NKp44 and NKp30 (activation receptors) which are distinct from counterparts in PB CD56^{bright} NK cells. There are similar accounts of trNK cell populations in lung, liver, and spleen (Cuff et al., 2016; Hudspeth et al., 2016; Lugthart et al., 2016; Lünemann et al., 2013; Marquardt et al., 2015, 2017; Stegmann et al., 2016; Yeang et al., 2017). In general, trNK cells do not express CD62L or CCR7, which are known to be highly expressed in PB CD56^{bright} NK cells. Instead, trNK cells express the adhesion molecule CD69, which inhibits S1P receptor to maintain tissue residence. In addition, trNK cells express chemokine ligands CCL3, CCL4, and CCL3L1, while having chemokine receptors CCR5 and CXCR6. In MALTs contiguous to epithelium, trNK will also express CD103 (integrin a_E) to bind E-cadherin (Cuff et al., 2016; Hudspeth et al., 2016; Lugthart et al., 2016; Stegmann et al., 2016).

This is not to say that trNK cell do not share similarities with PB CD56^{bright} NK cells. Much like CD56^{bright} NK cells, trNK cells exhibit poor cell-mediated cytotoxicity and robust cytokine production in response to monokines (if a little less than PB counterparts). By the same token, trNK cells also highly express CD94/NKG2A and NKp46 with low levels of markers associated with PB CD56^{dim} NK cells such as CD16, CD94/NKG2D, CD57, and KIRs (Freud et al., 2017; Melsen, Lugthart, Lankester, & Schilham, 2016).

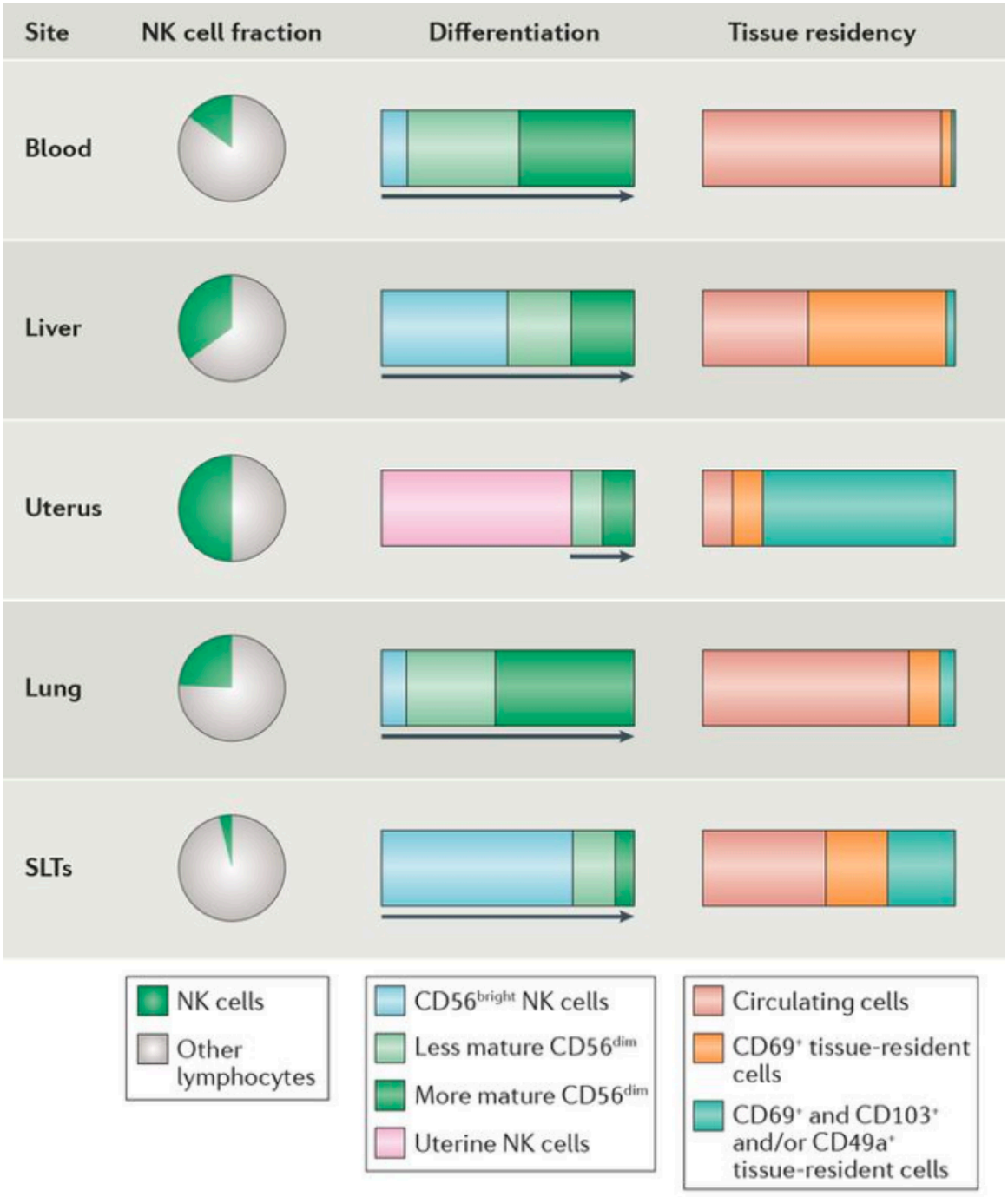

Figure 6. Distinguishing features of PB and tissue-resident NK cells. Relative proportion of NK cells in various compartments varies according to tissue type, as do markers of tissue residency and maturity. Adapted from (Björklund et al., 2016).

CD56^{dim} NK cells have similarly undergone closer inspection with result that various "adaptive" NK cells populations have been observed. While the details of NK cell immunological memory remain fairly nebulous, so-called "memory" NK cells have been known to arise in the context of human cytomegalovirus (HCMV) infection. These "adaptive" NK cells express similar levels of CD56 to PB CD56^{dim} NK cells, but exhibit relatively increased expression of CD57, CD2, and immunoglobulin-like transcript 2 (ILT2) with relatively decreased expression of CD7, CD161, NKp30, and NKp46 (Hwang et al., 2012; J. Lee et al., 2015; Schlums et al., 2015).

Because PB NK subsets are the most easily accessed in human subjects and have already been applied in NK cell immunotherapies, later discussion of trNK cells and "adaptive" NK cells will be limited (Barrow & Colonna, 2019; Davis, Felices, Verneris, & Miller, 2015). However, given the newfound abundance of these in cells in peripheral tissues, the investigation of non-conventional cells remains an active area of research (Figure 6).

B. Effector Functions of NK cells

As previously stated, NK cells have two major effector functions: cytokine production and cell-mediated cytotoxicity. Performing either of these functions first requires the integration of activating and inhibitory signals, which can originate from a variety of cell surface receptors (Vivier et al., 2008). This section will discuss how NK cells are activated, how they are inhibited, and how they "pull the trigger" when fulfilling their primary roles in immunity.

1. Activating/Inhibitory Receptors and Early Signal Transduction in NK Cells

Since NK cells were discovered, it was well-known that these lymphocytes could kill virally-infected or transformed cells. What was not known was how NK cells avoided killing normal cells. After a decade of research, the answer was revealed in a seminal paper published in 1986. In it, Karre and colleagues demonstrated that NK cells killed tumor cells deficient in major histocompatibility complex (MHC) class I, yet ignored those tumor cells expressing MHC class I (Kärre, Ljunggren, Piontek, & Kiessling, 1986). Given that the MHC class I is expressed by virtually all normal cells, (but tends to be downregulated in infected and transformed cells) this precipitated the "missing-self" hypothesis: abnormal cells simply lacked MHC class I, so in the absence of inhibitory signals, NK cells killed them (Lewis L. Lanier, 2004). However, that was only part of the story. The other part was that NK cells also have activating receptors whose ligands are upregulated during times of cellular stress. In fact, with enough activating signals, inhibition mediated by MHC class I expression could be overcome (Cerwenka, Baron, & Lanier, 2001; Lewis L. Lanier, Corliss, & Phillips, 1997). With additional data, the modern conception of NK cell activation expands the "missing-self" hypothesis: NK cells target abnormal cells deficient in "self" proteins (e.g. MHC class I) and/or overexpressing activating ligands (Lewis L. Lanier, 2004).

This thesis has already mentioned several activating receptors such as CD16, NKG2D, CD94/NKG2C, 2B4 (inhibitory in some circumstances), and DNAM-1 in the context of expression profiles of $CD56^{dim}$ vs. $CD56^{bright}$ NK cells. Other important

activating NK cell receptors include the natural cytotoxicity receptors (NCRs: NKp30, NKp44, and NKp46) (Bodduluru, Kasala, Madhana, & Sriram, 2015; Lewis L. Lanier, 2004, 2008). Of these, the NCRs and NKG2D are most important for anti-tumor activity (James, Cohen, & Campbell, 2013; Koch, Steinle, Watzl, & Mandelboim, 2013). In fact, NCR1 (NKp30) has been shown to be downregulated in patients with high-grade squamous intraepithelial lesions, cervical cancer, and metastatic neuroblastoma (NB) (Garcia-Iglesias et al., 2009; Semeraro et al., 2015). Even more, the NKp30 isoform can be predictive of clinical outcome in patients with gastrointestinal sarcoma and neuroblastoma (Delahaye et al., 2011; Semeraro et al., 2015).

CD16 is also relevant to anti-tumor activity, as it mediates ADCC by binding the Fc portion of IgG. Uniquely, CD16 is the only receptor whose activation can strongly induce NK cell cytotoxicity without a coactivating receptor. All other activating receptors (e.g. NKG2D, NKp30) require activation of a coreceptor of a different class to effectively overcome inhibitory stimuli (Bryceson, March, Ljunggren, & Long, 2006). Not all pairings are effective though. For example, NKG2D and DNAM-1 activate but cannot stimulate a robust cytotoxic response, while the combinations 2B4:NKG2D and 2B4:DNAM-1 can (Bryceson, Ljunggren, & Long, 2009). This synergy can be understood in terms of downstream signal transduction discussed later.

Table 2. Major Activating Receptors and their Reported Ligands in Human NK Cells. Adapted from (Bodduluru et al., 2014).

Receptor (alternate names)	Reported ligand (s)
Activating receptors	
CD2 (LFA-2)	CD58 (LFA-3)
CD7 (LEU-9)	SECTM1, galectin
CD11a (LFA-1)	ICAM-1/2/3/4/5
CD11b (Mac1, αMβ2, c3R)	ICAM-1/2, fibrinogen
CD16 (FCγRIII)	Fc of IgG immune complexes
CD28	B7-1/2
CD44	Hyaluronan
CD59 (Protectin)	C8,C9
CD69 (CLEC2C)	Unknown
CD94/CD159c (NKG2C); CD94/NKG2E	HLA-E
CD96 (TACTILE)	CD155
CD158h (KIR2DS1)	Group 2 HLA-C
CD158j (KIR2DS2, nkat5); KIR2DS3 (nkat7)	HLA-C
CD158i (KIR2DS4, nkat8)	Some HLA-C1 and HLA-C2, HLA-A11
CD 158j (KIR2DS5, nkat9)	Unknown
CD158d (KIR2DL4)	HLA-G
CD158e2 (KIR3DS1, nkat10)	HLA class I
CD160 (BY55)	HLA-C
CD223 (Lag3)	HLA class II
CD226 (DNAM-1)	Nectin-2, PVR (CD112, CD155)
CD314 (NKG2D)	MICA/B, ULBP1, ULBP2, ULBP3, ULBP4, ULBP5, ULBP6
CD319 (CRACC)	CRACC
CD335 (NKp46, NCR1)	HSPG, heparin, VM, HA (IV, VV, ECTV), HN (SeV, NDV), PfEMP-1, Fusobacterium nucleatum
CD336 (NKp44, NCR2)	PCNA, HSPG, heparin, E-protein (DV, WNV), HA (IV, SeV), HN (NDV), Mycobacterium, Nocardia farcinica, Pseudomonas aeruginosa
CD337 (NKp30, NCR3)	B7-H6, BAT3, HSPG, HA (VV, ECTV), pp65 (HCMV), PfEMP-1
NTB-A	NTB-A
NKp80 (KLRF1, CLEC5C)	AICL receptor (activation-induced C-type lectin)
NKp65 (KLRF2)	KACL (keratinocyte-associated C-type lectin)

As alluded to earlier, activating receptors tend to bind "self" proteins upregulated during infection or malignant transformation. For example, the ligands of NKG2D are MHC class chain-related protein (MIC) A, MICB, and non-MHC class I molecules including UL16-binding protein (ULBP(1-6)), all of which are upregulated in tumor cells (Bauer et al., 1999; Cosman et al., 2001). Moreover, NCRs bind to heparan sulfate on tumors and NKp30 specifically recognizes stress-induced B7H6 (Koch et al., 2013). A more exhaustive inventory of activating receptors is listed in Table 2.

With regard to signal transduction, most of the well-understood activating receptors are associated with immunoreceptor tyrosine-based activation motif (ITAM)-bearing molecules such as FcR γ chain, T cell receptor (TCR) ζ chain, DNAX-activating protein (DAP)12 (Figure 7). For example, NKp46 and NKp30 associate with FcR γ chain and/or the TCR ζ chain, NKp44 associates with DAP12 (a homodimer with a single ITAM). Meanwhile, NKG2D associates with a protein similar to DAP12, yet lacking an ITAM region—DAP10. In addition, CD16 associates with heterodimers and homodimers formed by the FcR γ chain and TCR ζ chain (Lewis L. Lanier, 2004; Moretta & Moretta, 2004). ITAMs have two tyrosines, which are phosphorylated by members of the Src-kinase family. Once phosphorylated, these ITAMs can be then bind the Src-homology domain 2 (SH2) regions of ZAP70 (Zeta chain of TCR associated with Protein 70) and Syk tyrosine kinases (Lewis L. Lanier, 2009; Watzl & Long, 2010). These kinases phosphorylate transmembrane adaptor molecules LAT (Linker for Activation of T cells) and NTAL (Non-T cell Activation Linker) which lead to association and subsequent phosphorylation of phosphoinositide 3 kinase (PI3K), phospholipase C (either PLC-γ1 or

PLC-γ2), and Vav2 or Vav3 (Marina Cella et al., 2004; Upshaw, Schoon, Dick, Billadeau, & Leibson, 2005). In contrast, DAP10 has a distinct signaling function and preferentially couples to PLC-γ2 as phosphorylated DAP10 binds either the p85 subunit of PI3K or the adaptor Grb2 associated with Vav1 (a guanine nucleotide exchange factor (GEF)) (Lewis L. Lanier, 2009, p. 10; Long, Kim, Liu, Peterson, & Rajagopalan, 2013; Long et al., 2013; Upshaw et al., 2005). Another signaling pathway worth mentioning is mediated by the immunoreceptor tyrosine-based switch motif (ITSM), which is relevant for members of the signaling lymphocytic activation molecule (SLAM) family such as 2B4 and CD2-like receptor activating cytotoxic cells (CRACC) (Veillette, 2010). SLAM receptors usually transmit activation signals through SLAM-associated protein (SAP). SAP both recruits Src-family kinase Fyn (which phosphorylates Vav1) and competes with the inhibitory SH2 domain-containing inositol 5' phosphatase-1 (SHIP-1) when binding 2B4 (Veillette, 2010). This second role of SAP partially explains the inhibitory behavior that 2B4 may sometimes exhibit. Either increased expression of 2B4 or decreased expression of SAP leads to greater activity of SHIP, SHP1, and SHP2, which inhibits NK activation (Eissmann et al., 2005; Sivori et al., 2002). Phosphorylated 2B4 may also associate with 3BP2, which when phosphorylated, interacts with LAT, Vav1, and PLC-γ (Saborit-Villarroya et al., 2005, p. 2). Meanwhile, SAP does not mediate CRACC signaling; it is instead mediated by a related adaptor protein called Ewing's sarcoma-associated transcript 2 (EAT-2), which does not bind Fyn. (Cruz-Munoz, Dong, Shi, Zhang, & Veillette, 2009).

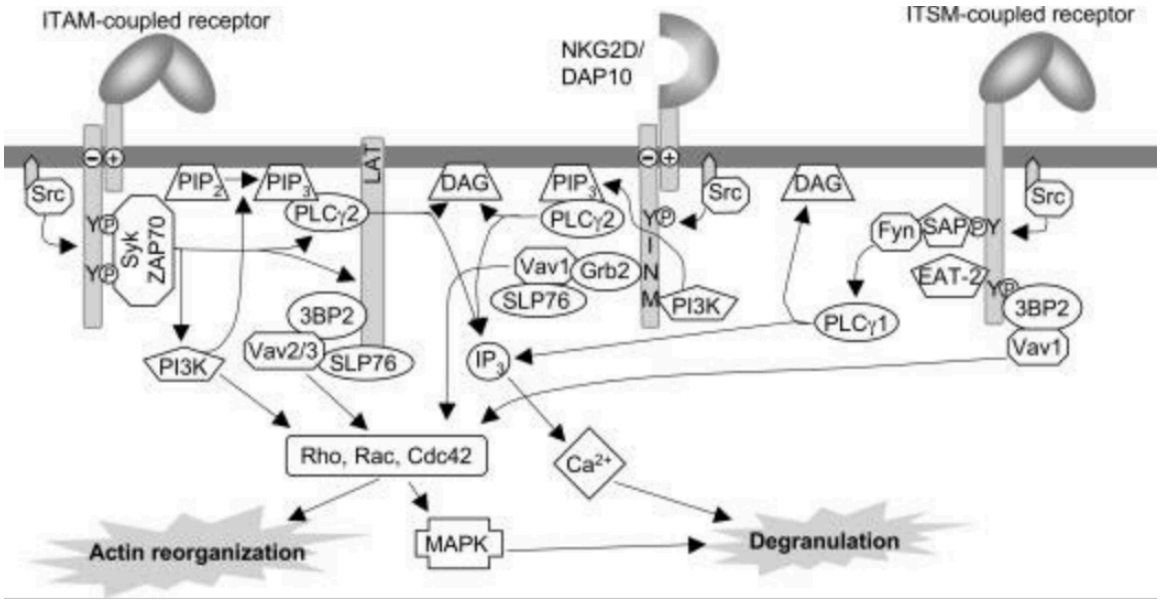

Figure 7. Activation Pathways in NK Cells. There are three main schemes for signal transduction following binding of extracellular ligands to activating receptors: ITAM-coupled receptors (e.g. NCRs/DAP12), NKG2D/DAP10, and ITSM-coupled receptors (e.g. SLAM receptors). Adapted from (Watzl & Long, 2010).

Now that some of receptor signal transduction has been elucidated, it will be easier to explain the particular complementary pairings necessary for NK cell activation. The receptor complementarity appears to depend on the selective phosphorylation of two tyrosine residues on SLP76 (Figure 8) (H. S. Kim & Long, 2012). 2B4 activation leads to preferential phosphorylation of Tyr 113 while NKG2D and DNAM-1 tend to phosphorylate Tyr 128. These phospho-tyrosines can then bind Vav1. Unsurprisingly, CD16 signaling results in the phosphorylation of said tyrosines, which may explain why CD16 can induce NK cell cytotoxicity without a coactivating receptor (H. S. Kim & Long, 2012; Long et al., 2013).

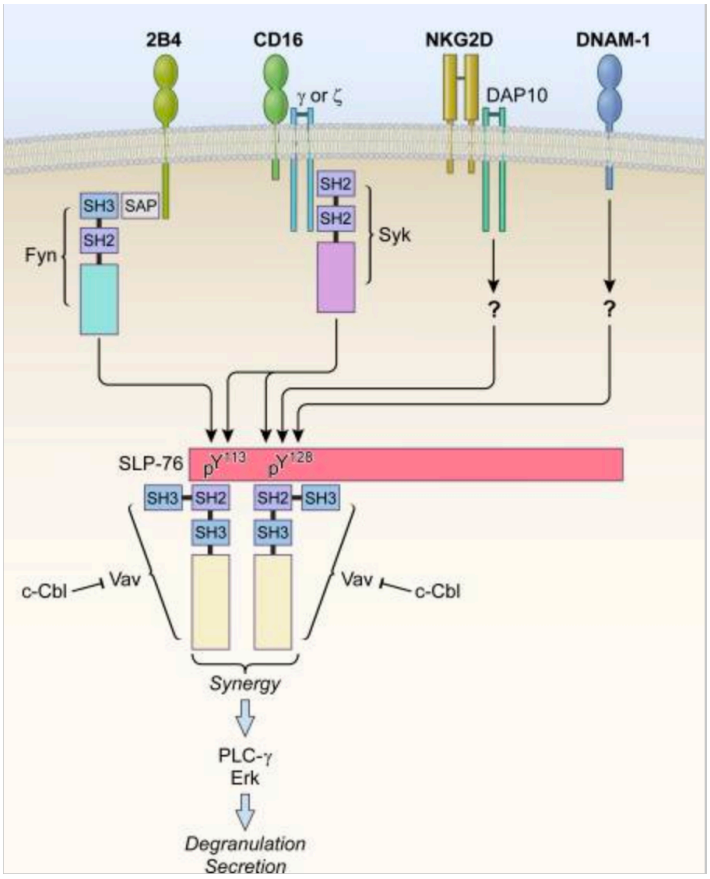

Figure 8. Synergistic Signaling in NK Cell Activation. Post-receptor signaling converges on SLP-76, which may integrate complementary signals to activate NK cells. Note that c-Cbl is an ubiquitin ligase. Adapted from (Long et al., 2013).

Meanwhile, inhibitory receptors include the superfamily of KIRs (a minor subset of which is activating due to association with DAP12), leukocyte immunoglobulin-like receptors (ILT2) and type II glycoproteins with a C-type lectin scaffold such as CD94/NKG2A (Bodduluru et al., 2015; Lewis L. Lanier, 2004). Each of these receptors binds a different human leukocyte antigen (HLA) class. KIRs binds HLA-A, HLA-B, and

HLA-C while CD94/NKG2A binds non-classical HLA-E. Ligands for ILTs include the aforementioned HLA classes in addition to HLA-G and HLA-F (Bodduluru et al., 2015; Joyce & Sun, 2011).

Just as several activating receptors have an ITAM sequence, major inhibitory receptors possess an immunoreceptor tyrosine-based inhibition motif (ITIM), which is critical to early termination of activation signals. Specifically, engaged ITIM-bearing receptors recruit tyrosine phosphatases SHP-1 and SHP-2 via SH2 domain binding (Burshtyn et al., 1996; Burshtyn, Yang, Yi, & Long, 1997). SHP-1 has been shown to dephosphorylate Vav1, a protein known to be important in the activation cascade (Figure 7 and Figure 8) (Peterson & Long, 2008). Inhibition in NK cells (when targeting an MHC-I expressing cell) also corresponds with the adaptor protein Crk (CT10 regular of kinase) becoming phosphorylated and associating with tyrosine kinase c-Abl (Peterson & Long, 2008). Crk—required for CD16 signaling—links the GEF C3G to cytoskeletal scaffold proteins. Crk phosphorylation results in dissociation from the C3G:cCbl complex, which likely contributes NK cell inhibition (Chodniewicz & Klemke, 2004; Liu, Peterson, & Long, 2012). Inhibitory signaling also blocks "inside-out" and "outside-in" signaling from integrin LFA-1, known to facilitate adhesion and granule polarization at the immunological synapse (i.e. contact point between NK cell and target cell) (Barber, Faure, & Long, 2004; Bryceson et al., 2009). Moreover, inhibition tends to dominate activation signals, as shown by live-imaging in which inhibitory receptor microclusters formed within seconds, immediately suppressing formation of activating receptors microclusters (Abeyweera, Merino, & Huse, 2011; Watzl & Long, 2003, p. 4).

Much of NK cell receptor signaling has been investigated by isolating different pathways with reductionist approaches. Meanwhile, understanding the integration of activating and inhibitory signals from extracellular ligands still proves incredibly challenging (Long et al., 2013). Whereas T and B cells have distinct signaling components, NK cells incorporate features of both B and T cells, which adds an additional layer of complexity (Watzl & Long, 2010). Indeed, the complexity in receptor signaling goes even further. While NK cells receptors do not require genetic rearrangement via RAG-based mechanisms like their adaptive counterparts, NK cell receptors still manage to display profound functional diversity (Bjorkstrom, Ljunggren, & Michaelsson, 2016). In other words, each NK cell expresses a different collection of functional receptors. In fact, KIRs exhibit clonal distribution patterns mediated by epigenetic regulation. This and the unusual polymorphism of KIRs result in highly stochastic receptor repertoires with varying degrees of responsiveness to different target cells (Bjorkstrom et al., 2016; Manser, Weinhold, & Uhrberg, 2015). This proves particularly relevant in NK cell "education," which is discussed next.

2. NK Cell Education

MHC class I specific inhibitory receptors such as KIRs do more than send inhibitory signals. Self-recognizing inhibitory receptors (SRIR) participate in a phenomenon known as NK cell "education" (Campbell & Hasegawa, 2013). This line of investigation emerged from early studies in β_2-microglobulin deficient (i.e. deficient in MHC class I) mice in which autologous NK cells did not kill autologous MHC class I

deficient cells (Höglund et al., 1991; Liao, Bix, Zijlstra, Jaenisch, & Raulet, 1991). This result challenged the "missing self" hypothesis, which predicted hyper-responsive NK cells in the absence of inhibitory signals. On the contrary, NK cells native to MHC class I-deficient environments were *hypo-responsive* to activation signals. The same was true of NK cells lacking inhibitory receptors (Fernandez et al., 2005; Sungjin Kim et al., 2005). To explain this startling self-tolerance, several models have since been proposed (Figure 9). Licensing or arming is a model that suggests that ITIM-bearing receptors signal in a way that prepares NK cell machinery for activation stimuli (Sungjin Kim et al., 2005) Whether or not these signals are distinct from those important for NK cell inhibition is unclear (Long et al., 2013). In contrast, the disarming model focuses more on active receptors. It explains that chronic activation in the setting of MHC class I deficiency leads to hypo-responsiveness in a way similar to anergy of T cells. In that scenario, ITIM signaling "rescues" the NK cell from this anergic state (Raulet & Vance, 2006). The rheostat model rejects a dichotomy of "educated" vs "non-educated" in favor a quantitative model. It postulates that the number or density of SRIR positively correlates with NK effector potential. The stronger the inhibition signals, the more responsive the NK cell (Brodin, Lakshmikanth, Johansson, Kärre, & Höglund, 2009; Joncker, Fernandez, Treiner, Vivier, & Raulet, 2009; Narni-Mancinelli et al., 2012; Junli Yu et al., 2007). Lastly, the "tuning" model takes the "rheostat" further, suggesting that not only is NK cell education quantitative, but there is an inherent plasticity in degree of education (Brodin et al., 2009; Pradeu, Jaeger, & Vivier, 2013). Put simply, an NK cell can become or less responsive based on MHC class I environment and/or SRIR

expression. More explicitly, NK cells are tuned to the MHC class I environment and only kill cells with sudden decreases in MHC class I expression (Long et al., 2013).

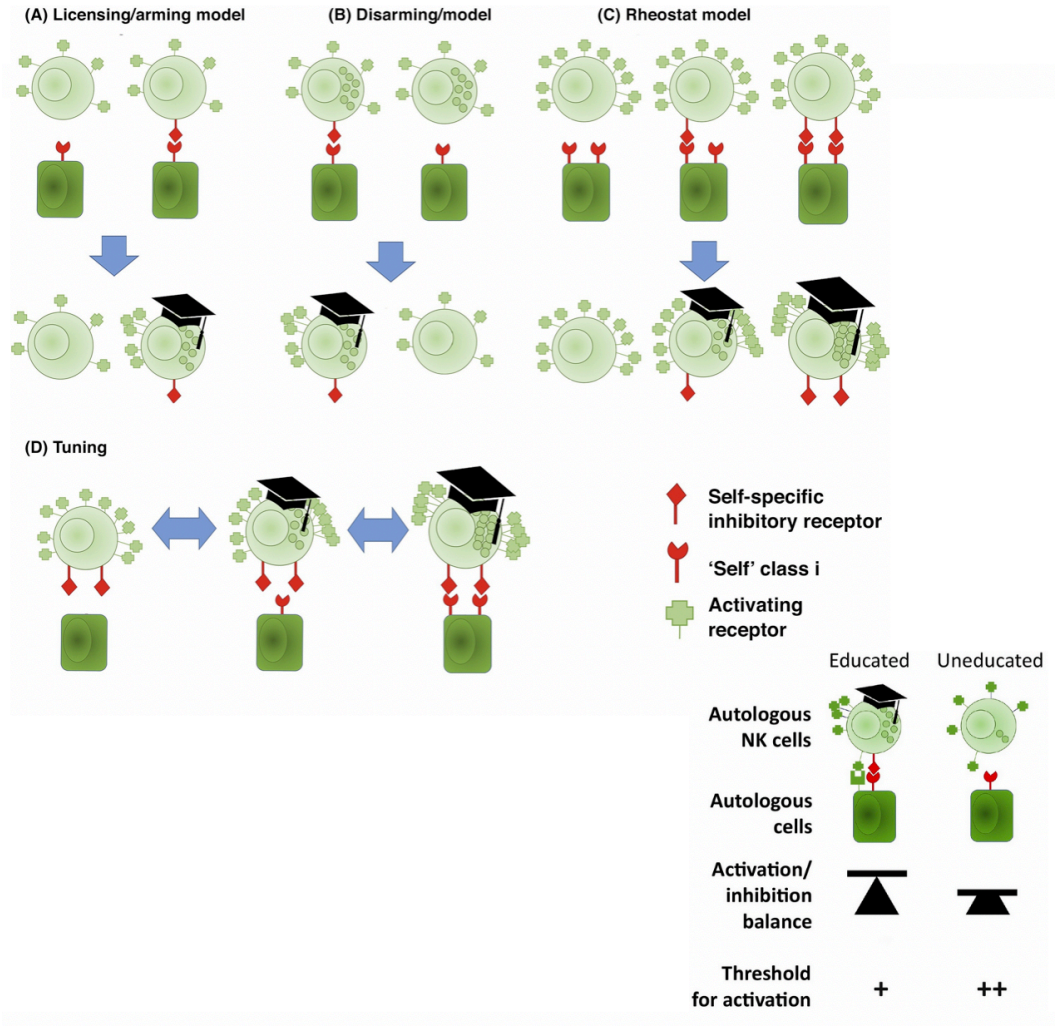

Figure 9. Models of NK Cell Education. (A) Licensing/Arming: NK cells initially have low effector potential that increases with inhibitory signals. (B) Disarming: NK cells have high effector potential but chronic activation leads to exhaustion and anergy. Inhibitory signaling "rescues" effector potential. (C) Rheostat: avidity of SRIR binding and signaling corresponds to subsequent NK cell effector potential. (D) Tuning: consistent with other models, but contends that effector potential is a transient property that can adjust during life of NK cell depending on circumstances. Adapted from (Boudreau & Hsu, 2017).

While the molecular mechanism of NK cell education remains unclear, Long et al recently proposed that Crk could mediate at least one part. In murine models, licensed or "educated" NK cells display decreased confinement of activating receptors. Recall from the last section that Crk binds scaffold proteins in the cytoskeleton, but when phosphorylated, Crk dissociates from this scaffold. It is possible that Crk phosphorylation—though inhibitory— would disrupt the filamentous actin (F-actin) network, thereby removing the restriction on movement of activating receptors. Ultimately, repeated inhibitory signaling might promote microclusters of activating receptors upon subsequent activation. On the other hand, chronic activation with unphosphorylated Crk would establish a restrictive F-actin network (Long et al., 2013). While this hypothesis agrees with most of the education models, additional data is needed.

It is worth mentioning that there may still be a role for "uneducated NK cells." Unlicensed SRIR-negative NK cells have been shown *in vivo* to respond more strongly than SRIR-positive NK cells in the setting of murine cytomegalovirus infection (MCMV) (Orr, Murphy, & Lanier, 2010). Even more, the functionality of SRIR-deficient NK cells can be partially recovered with IL-2 supplementation (Sungjin Kim et al., 2005).

3. Cell-mediated Cytotoxicity

NK cells can directly kill target cells in two major ways: with granule exocytosis and death receptor-induced apoptosis (Chester, Fritsch, & Kohrt, 2015). The first mechanism requires the formation of an immunological synapse, through which NK cells

exocytose secretory lysosomes containing perforin and granzymes (Clark & Griffiths, 2003). Perforins "perforate" or otherwise disrupt membrane integrity of the target cell, while granzymes are serine proteases that induce apoptosis by activating caspase-dependent or caspase-independent pathways (J. Lieberman, 2003; Pinkoski et al., 2001; Thiery et al., 2011). Strictly speaking though, a recent study has shown that NK cells may induce either necrosis, apoptosis, or "mixed forms" with granule exocytosis as well. Moreover, the ratio of necrosis to apoptosis could be increased with greater concentrations of perforin or extracellular Ca^{2+} (Backes et al., 2018). In contrast, death receptor ligation exclusively induces apoptosis. This mechanism relies on target cell expression of receptors in the tumor necrosis factor (TNF) superfamily. The TNF receptors most relevant to induction of apoptosis by NK cells are FasR (Fas receptor) and the receptor for TNF-related apoptosis-inducing ligand (TRAIL). Both these TNF receptors cause pro-apoptotic signaling through caspase activation (Falschlehner, Emmerich, Gerlach, & Walczak, 2007; Kerr, Wyllie, & Currie, 1972). While FasR enjoys widespread expression on a variety of tissues, TRAIL receptors (TRAILR1 and TRAILR2) are less common (Mark J. Smyth et al., 2005; Yagita, Takeda, Hayakawa, Smyth, & Okumura, 2004). Moreover, Fas ligand (FasL) is only expressed on activated NK cells and cytotoxic T lymphocytes (CTLs) , while some NK cell populations constitutively express TRAIL regardless of activation status (Mark J. Smyth et al., 2005; Takeda et al., 2001; Yagita et al., 2004). Given the severe immune disorders that result from defects in the machinery of exocytosis, perforin/granzyme secretion is considered to be the primary mechanism of direct NK cell cytotoxicity (Arneson et al., 2007, p. 11; A.

Fischer, Latour, & de Saint Basile, 2007; Topham & Hewitt, 2009). Consequently, this thesis will describe this mechanism in greater detail.

Because of its complexity, it can be useful to divide granule exocytosis into four stages. The first stage was alluded to into in earlier section. Namely, a lytic immunological synapse (as opposed to the other non-lytic immunological synapse that sometimes forms between NK cells and other immune cell types) forms and the actin cytoskeleton rearranges at the point of contact. Second, the lytic granules and microtubule-organizing center (MTOC) polarize towards the aforementioned synapse. Third, the secretory lysosomes containing cytotoxic proteins dock at the cell membrane. And last, the lysosomes fuse with cell membrane to exocytose contents. Figure 10 illustrates these four stages (Topham & Hewitt, 2009).

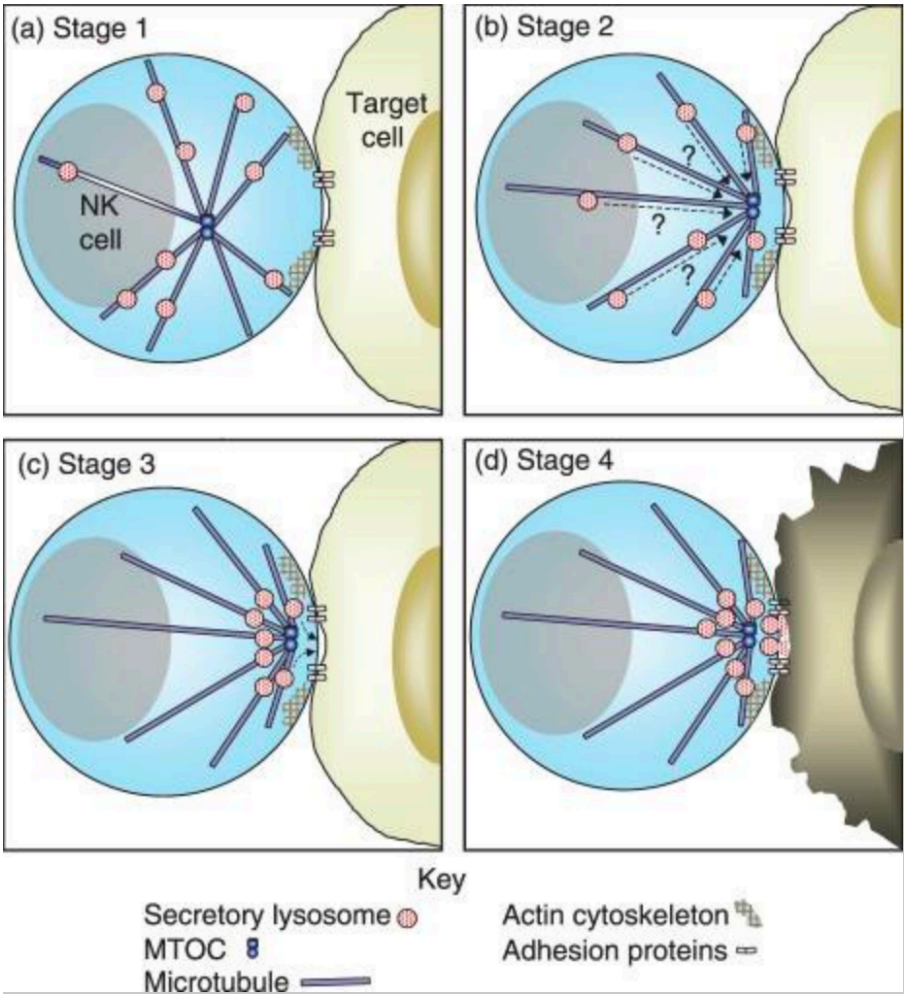

Figure 10. Granule Exocytosis in NK Cells. It is useful to consider secretory lysosome exocytosis in four stages. a) Lytic immune synapse formation with F-actin ring assembly. b) *MT*OC polarization to immune synapse. It is unclear if secretory lysosomes move along microtubules or move via another method. c) Secretory lysosomes "dock" next to plasma membrane. d) Secretory lysosomes fuse with cell membrane at the immune synapse and release contents. Adapted from (Topham & Hewitt, 2009).

Imagine that a synergistic combination of activating receptors (e.g. 2B4 and DNAM-1) are engaged. As mentioned before, in absence of strong inhibitory signals, the activating receptors will begin to cluster into a microdomain (Orange, 2007; Vyas et al., 2001). At this point of contact, two regions can be identified: the peripheral supramolecular activation cluster (pMAC) and the central supramolecular activation cluster (cMAC). The pMAC consists of integrins such LFA-1 in a ring while the cMAC is focal point of granule exocytosis. The cMAC allows NK cells to target cells without endangering other cells in close apposition. It is not well-understood how the signal transduction discussed earlier leads to polarized exocytosis of granules, however researchers have demonstrated that different signals may control different aspects of the process as a whole. For instance, the LFA-1 ring in the pMAC produces signaling which leads to lysosome polarization, but not exocytosis (i.e. degranulation). In contrast, CD16 activation causes degranulation, but cannot induce granule polarization (Barber et al., 2004; Bryceson, March, Barber, Ljunggren, & Long, 2005). The uncoupling of polarization and degranulation likely provides an additional safeguard against unrestricted NK cell cytotoxicity.

Actin polymerization/depolymerization and cytoskeletal rearrangement are critical to polarized exocytosis (Topham & Hewitt, 2009). During lytic immune synapse formation, F-actin accumulates in the pMAC through action of the Wiskott-Aldrich syndrome protein (WASp) (Orange et al., 2003, 2002). Moreover, WASp modulates the actin cytoskeleton through Arp2/3 to control nucleation and branching (Stradal et al., 2004). Note that WASp can only interact with Arp2/3 complex when WASp binds GTP

bound Cdc42 (a GTPase). Given that the Vav1 can be a GEF for Cdc42 (see Figure 7), WASp may very well be a link between NK receptor signaling and polarized degranulation (Gismondi et al., 2004; Watzl & Long, 2010).

The precise mechanics of MTOC and secretory lysosome polarization are not well-characterized in NK cells though researchers suspect many similarities with related phenomena studied in CTLs (Topham & Hewitt, 2009). MTOC is thought to precede polarization of secretory lysosomes. And it has been demonstrated in NK cells that failure of MTOC to localize at lytic synapse precludes polarization of secretory lysosomes. This may be mediated through Cdc42-interacting protein 4 (CIP4), which interacts with microtubules and co-localizes with WASp and the MTOC at the lytic immune synapse (Banerjee et al., 2007). As for granule polarization, WASp-interacting protein (WIP) and the GTPase Rab7 may have roles. WIP interacts with secretory lysosomes and forms a complex with WASp, F-actin, and Myosin IIa that later moves to the lytic immunological synapse (Krzewski, Chen, & Strominger, 2008). Along the same lines, Rab7 has a known role in the movement of conventional lysosomes along microtubules through effector Rab7 interacting lysosomal protein (RILP) (Cantalupo, Alifano, Roberti, Bruni, & Bucci, 2001). In fact, RILP overexpression results in increased clustering of secretory lysosomes near the MTOC in CTLs (Stinchcombe, Majorovits, Bossi, Fuller, & Griffiths, 2006). While the role of Rab7 and RILP is less clear in NK cells, researchers have identified Rab7 in secretory lysosome fractions of the human NK cell line YTS (Casey, Meade, & Hewitt, 2007).

For the docking of secretory lysosomes, two proteins deserve attention: Rab27a and Myosin IIa. Recall that Myosin IIa formed a complex with WIP, WASp, and F-actin early in stage 2 during polarization of MTOC (Krzewski et al., 2008). Notably, while Myosin IIa depletion has no effect on polarization of MTOC or secretory lysosomes, its depletion does correspond with decreased ability of lysosomes to fuse with the plasma membrane (Andzelm, Chen, Krzewski, Orange, & Strominger, 2007; Ito et al., 1989). Likewise, the GTPase Rab27a seems to be associated with lysosome docking/fusion. In CTLs, mutated Rab27a leads to successful polarization of secretory lysosomes without fusion with plasma membrane. Similarly, NK cells with nonfunctional Rab27a have impaired NK cell cytotoxicity in the setting of NKp30 stimulation. However, CD16 induced cytotoxicity remains unaffected (Gazit et al., 2007). This potentially implies two pathways: one Rab27a-dependent, the other Rab27a-independent.

Relying again on data from CTLs, the Rab27a effector protein Munc13-4 has been shown to be required for fusion of secretory lysosomes in CTLs (Feldmann et al., 2003; Neeft et al., 2005). Electron microscopy of CTLs with mutated Munc13-4 reveals that secretory lysosomes were able to dock, but could not fuse with the cell membrane (Feldmann et al., 2003). Given that both CTLs and NK cells in patients with mutated Munc13-4 both demonstrate impaired cytotoxicity, it stands to reason that Munc13-4 may have similar roles in both types of cytotoxic lymphocytes (Feldmann et al., 2003; Marcenaro et al., 2006). How Munc13-4 promotes membrane fusion is still not clear. However, in general soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) catalyze membrane fusion (Jahn & Scheller, 2006). Given that a

homologue of Munc13-4—Munc13-1—promotes membrane fusion by releasing syntaxin 1A from Munc18 so that syntaxin 1A can form a SNARE complex, one might hypothesize that Munc13-4 acts similarly (Gladycheva, Ho, Lee, & Stuenkel, 2004; Topham & Hewitt, 2009).

The SNAREs involved in secretory lysosome fusion are relatively unknown. However, the mutation of syntaxin 11 does severely impair lysosome exocytosis and associated NK cell cytotoxicity (Bryceson et al., 2007; zur Stadt et al., 2005). This impairment can be overcome though with prolonged culture in IL-2, suggesting the existence of syntaxin 11 dependent and independent pathways in SNARE-mediated fusion of lysosomes (Bryceson et al., 2007). Other SNAREs associated with NK cell secretory lysosomes include vesicle-associated membrane protein 7 (VAMP7) and syntaxin 7 (Casey et al., 2007).

Though dozens of proteins have been implicated in the exocytosis of secretory lysosomes for NK cells, there is still much inferred from CTLs. And while one might expect cytotoxic lymphocytes to display similar mechanisms of lysosome exocytosis, the known differences between CTLs and NK cells call this into question. Much remains to be elucidated, especially with regard to the integration of receptor signaling. Undoubtedly, a more complete understanding the complex machinery of lysosomal exocytosis will empower researchers to pinpoint what may precisely affect NK cell cytotoxicity in cancer, immune disorders, and other pathological conditions.

4. Cytokines: Roles in NK Cell Effector Functions

In addition to the direct cytolysis mediated by NK cells, these lymphocytes can also mediate indirect cell killing through the production of cytokines. Through cross-talk with and cytolysis of other immune cells such as B cells, DCs, macrophages, and T cells, NK cells play a pivotal role in the course of innate/adaptive immune responses (Figure 11) (Vivier et al., 2011).

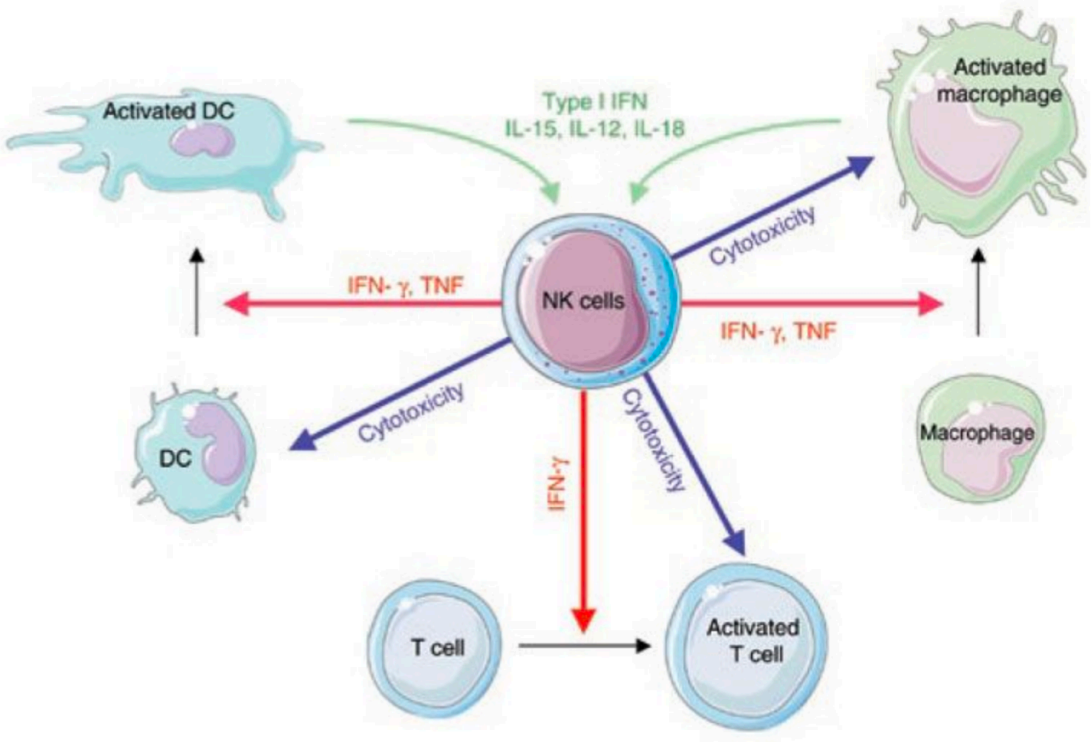

Figure 11. NK Cell Interactions with Other Immune Cells. NK cell production of IFN-γ, and TNF (tumor necrosis factor) induce activation of DCs, macrophages, and T cells. In turn, these diverse cell types produce pro-inflammatory cytokine, creating a positive feedback loop. Conversely, NK cells may also direct cytotoxic granules to activated macrophages, activated T cells, and immature macrophages to fulfill an anti-inflammatory role. Adapted from (Vivier et al., 2008).

While NK cells can produce a variety of pro-inflammatory and immunosuppressive cytokines, NK cells tend to produce Th1-associated cytokines such as IFN-γ, TNF-α, and granulocyte/monocyte colony-stimulating factor (GM-CSF) in response to tumor ligands or intracellular pathogens (Abel et al., 2018; Cook, Waggoner, & Whitmire, 2014, p. 10; Vivier et al., 2011). Much like the induction of direct cytotoxic behavior in NK cells, stimulation of activating receptors mentioned earlier (e.g. NKG2D, 2B4, DNAM-1) can induce pro-inflammatory cytokine production. This effect is most profound in $CD56^{dim}$ NK cells, as this subset secretes significantly more cytokines upon target cell recognition than most $CD56^{bright}$ NK cells (traditionally considered more active cytokine producers) (Fauriat et al., 2010). Different sets of activating receptors correspond with production to different cytokines. For example, chemotactic cytokines (i.e. "chemokines"), which attract other immune cells, require fewer activating receptors to stimulate secretion than for type I cytokines (e.g. IFN-γ, TNF) (Blanchard, Michelini-Norris, & Djeu, 1991; Fauriat et al., 2010; Walzer, Dalod, Robbins, Zitvogel, & Vivier, 2005). Engagement of NKG2D, CD16, or 2B4 induced secretion of chemokines such as CCL3 (macrophage inflammatory protein (MIP)-1α), CCL4 (MIP-1β), CCL5 (regulation on activation, normal T cell expressed and secreted (RANTES)). In contrast, TNF-α release has been shown to require coactivation of NKG2D and 2B4 while IFN-γ release has been shown to require coactivation of LFA-1, NKG2D, and 2B4 (Fauriat et al., 2010). Additionally, chemokine release was shown to precede the release of type I cytokines, consistent with a model in which NK cells first attract immune cells to maximize pro-inflammatory effects (Fauriat et al., 2010).

It should be noted that though stimulation of NK cell activating receptors can induce secretion of both cytotoxic granules and cytokines, the delivery pathways are quite distinct (Reefman et al., 2010). Relatively little is known about the cytokine release in NK cells, though recently these cytokines have been found to co-localize with "recycling endosomes"—a central organelle in cytokine secretion of macrophages. Moreover, cytokine release does not occur in a polarized fashion (R. Z. Murray, Kay, Sangermani, & Stow, 2005; Reefman et al., 2010). Because of the divergent secretory pathway, it may not be surprising that cytokine and lytic granule release are mediated by different signaling pathways. In 2013, Rajasekaran and colleagues demonstrated that while NK cell cytotoxicity and cytokine production both required Lck, Fyn, PI3K, and PLC, interaction between Fyn and Adhesion and Degranulation-promoting Adaptor Protein (ADAP) was critical to cytokine production (Rajasekaran et al., 2013) Moreover, $ADAP^{-/-}$ NK cells had reduced nuclear translocation of transcription factors such as c-Jun, c-Fos, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65. Supporting the role of ADAP in cytokine production, these transcription factors bind promoters to enhance transcription of pro-inflammatory cytokine genes (Sica et al., 1997; Zhang et al., 1998). Meanwhile, another substrate of Fyn—Vav1—was shown to have to no effect on cytokine production, yet proves crucial NK cell-mediated direct cytotoxicity (Rajasekaran et al., 2013).

Exogenous cytokines (i.e. cytokine produced by DC and T cells) are just as relevant to NK cell effector function as endogenous cytokine production. Myriad cytokines such as IL-2, IL-15, IL-12, IL-18, and IL-21 among others critically enhance

NK cell cytotoxicity (Wu, Tian, & Wei, 2017). In view of this, the last part of this section will briefly review the effects of each cytokine on NK cells.

First identified as a growth factor for T cells, IL-2 derived from activated T cells has been shown to activate NK cells *in vitro* and improve NK cell response in infection (Fehniger et al., 2003; Malek, 2008; Trinchieri, 1989). Even more, IL-2 deficient mice demonstrate impaired NK cell activity, yet normal development (Kündig et al., 1993). In light of this, it is likely that IL-2 facilitates NK cell effector functions with no major role in NK cell ontogenesis (Wu et al., 2017). The receptor for IL-2 is heterotrimeric with subunits IL-2Rα (CD25), IL-2R/15Rβ (CD122) and IL-2Rγ (CD132, γ_c) and signal transduction occurs through the Janus kinase (JAK) 1/3-signal transducer and activator of transcription (STAT)5 pathway (Sim & Radvanyi, 2014). Two of the IL-2 receptor subunits—CD122 and CD132—are highly expressed by NK cells but the heterodimer CD25/CD122 and heterotrimeric receptor CD25/CD122/CD132 exhibit the highest affinities (Rickert, Wang, Boulanger, Goriatcheva, & Garcia, 2005; H. M. Wang & Smith, 1987). CD25 expression, however, can be induced with exposure to other cytokines such as IL-12, IL-15, and IL-18 (Leong et al., 2014; Ni, Miller, Stojanovic, Garbi, & Cerwenka, 2012). This proves particularly relevant in the setting of NK cell-based immunotherapies. The earliest trials infused IL-2 to improve NK cell response, which unexpectedly favored regulatory T cell (T reg) proliferation. *In vivo*, depletion of T reg cells or blocking CD25 expression on T cells seems to improve bioavailability of IL-2 for NK cells, thereby supporting IFN-γ production and cytotoxicity (Martin, Perry, Jakhete, Wang, & Bielekova, 2010; Sitrin, Ring, Garcia, Benoist, & Mathis, 2013).

IL-15 replicates most of the "immune-stimulatory" effects caused by IL-2 (Burton et al., 1994; Ma, Koka, & Burkett, 2006). In fact, IL-15 share more than effects with IL-2; their respective cytokine receptors share two subunits—CD122 and CD132 (Giri et al., 1994, 1995). Only the α subunits differ, where IL-15Rα has an affinity comparable to that of IL-2Rαβγ ($K_d = 10^{-11}$) (Giri et al., 1995). Consequently, low concentrations could possibly efficiently activate NK cells (Wu et al., 2017). Similar to IL-2 and most common γ chain cytokine receptors, IL-15 signals through the JAK-STAT5 pathway to mediate NK cell development and preserve viability (Bernasconi et al., 2006; Eckelhart et al., 2011, p. 1; Imada et al., 1998; Wu et al., 2017). IL-15 (usually "trans-presented" by DCs) also "primes" NK cells for secondary stimuli including other cytokines (Fehniger et al., 1999; Lucas, Schachterle, Oberle, Aichele, & Diefenbach, 2007). For instance, in IL-15-primed NK cells, IL-12 induces increased IFN-γ production (Nandagopal, Ali, Komal, & Lee, 2014). This priming effect is not limited to cytokine production though (Fehniger et al., 2007). Moreover, the priming effect depends on the IL-15-AKT-mTOR (mechanistic target of rapamycin) activation pathway (Ali, Nandagopal, & Lee, 2015; Nandagopal et al., 2014). Not only does inhibition of the PI3K-mTOR signal reduce the IL-12 induced phosphorylation of STATs in IL-15 primed NK cells, it also diminishes the rapid production of lytic granules after IL-15 exposure (Fehniger et al., 2007; Nandagopal et al., 2014). Remarkably, unlike IL-2, IL-15 primed NK cells remained highly functional despite cytokine withdrawal (Mao et al., 2016).

As mentioned before, IL-12 can induce robust production of IFN-γ (M. Kobayashi et al., 1989). Mainly produced by antigen-presenting cells (APCs) such as monocytes.

DCs, and macrophages, IL-12 engages the heterodimer IL-12Rβ_1/β_2 with signaling through tyrosine kinase 2 (TYK2) and JAK2-STAT4 (M. Cella et al., 1996, p. 40; Hunter, 2005; Kaplan, Sun, Hoey, & Grusby, 1996; Macatonia et al., 1995, p. 1; Trinchieri, Pflanz, & Kastelein, 2003). Some research groups have implicated IL-12 in the generation of "memory" NK cells, which are longer-lived and exhibit greater responses to activating stimuli (Megan A. Cooper et al., 2009; O'Leary, Goodarzi, Drayton, & von Andrian, 2006; Romee et al., 2012). In combination with IL-15 and IL-18, IL-12 pre-activated memory-like NK cells that display increased expression of CD25 with sensitivity to IL-2 at picomolar concentrations (Leong et al., 2014). IL-12 can also recover the functionality of uneducated NK cells (see Figure 9) (Sungjin Kim et al., 2005; Wagner et al., 2017). In fact, cytokine cocktails including IL-12, IL-15, and IL-18 can induce *de novo* expression of inhibitory receptors such KIR and CD94/NKG2A to facilitate education in a MHC class I competent environment (Juelke, Killig, Thiel, Dong, & Romagnani, 2009; Romagnani et al., 2007).

A member of the IL-1 cytokine family, IL-18 is produced by diverse cell types such as DCs, neutrophils, microglial cells, epithelial cells, and macrophages (Oertli et al., 2012; Okamura et al., 1995; Pizarro et al., 1999; Spörri, Joller, Hilbi, & Oxenius, 2008). IL-18 stimulates IFN-γ production through the heterodimeric receptor IL-18R1/18R accessory protein (which IL-12 treatment can upregulate) (Yoshimoto et al., 1998, p. 1). Signal transduction proceeds with myeloid differentiation primary response protein 88 (MyD88), which recruits IL-1R-associated kinase 4 (IRAK4) and TNFR-associated factor 6 (TRAF6). Recruitment of these mediators promotes activation of NF-κB and

mitogen-activated protein kinase (MAPK) which increase transcription of *IFNG* and stabilize the associated mRNA (Boraschi & Tagliabue, 2013; J.-K. Lee et al., 2004).

Produced by NKT cells, T follicular helper cells, and Th17 cells, IL-21 can also stimulate cytokine production and cytotoxicity in NK cells, especially when acting synergistically with cytokines previously described (e.g. IL-15, IL-2, IL-18). Notably, IL-21 treatment can reduce effects of IL-15 on NK cell proliferation and survival. Signaling occurs through an IL-18R/γ_c receptor complex which activates JAK1/3, thereby phosphorylating STAT1/3/5 (primarily STAT3). MAPK and PI3K/AKT pathways are also relevant to IL-21 signaling. Although so far this thesis has focused on activating cytokines, two cytokines associated with low cytotoxicity, tolerant NK cells will be discussed—transforming growth factor β (TGF-β) and IL-10.

An important immunoregulatory cytokine, several immune cell types secrete IL-10, including NK cells, B cells, T cells, DCs, and macrophages. Originally identified as an inhibitor of cytokine secretion in Th1 cells, IL-10 has been shown to maintain hypo-responsive NK cells in the liver—a chronic pathogenic environment. Moreover, intrahepatic IL-10 has been connected with NKG2A-dependent NK cell priming of DCs, which in turn induce T regulatory cells. IL-10 binds IL-10R which mediates signaling along the JAK1/TYK2-STAT1/3/5 axis (Moore, de Waal Malefyt, Coffman, & O'Garra, 2001, p. 10; Verma et al., 2016). Note this axis shares some mediators with a few pro-inflammatory cytokines despite its ultimately immunosuppressive effects. Meanwhile, TGF-β binds its receptors (primarily TGF-βRI and TGF-βRII), activating a cascade of Smad proteins which translocate to the nucleus and regulate gene expression. TGF-β

signaling negatively affects NK cell effector function in several ways. Namely, upregulation of TGF-β represses expression of the transcription factor T-bet (important for IFN-γ production and CD122 (also known as IL2Rβ which is important to IL-2/IL-15 signaling), reduces NKG2D/DAP10, diminishes 2B4/SAP signaling, and induces expression of microRNAs (miR)-146a and miR-183 (Donatelli et al., 2014; Sun et al., 2012; Viel, Besson, Marotel, Walzer, & Marçais, 2017; Xu, Han, Hou, Zhang, & Zhang, 2017; Jianhua Yu et al., 2006). MiR-146a has been shown to decrease NK cell cytotoxicity and IFN-γ secretion while miR-183 either inhibits DAP12, NKG2D, or NKp30 expression (Castriconi et al., 2003; Donatelli et al., 2014; Xu et al., 2017).

As shown above, cytokines serve major roles in NK cell biology. Clarifying their roles has been important to understanding NK cell effector function *in vivo* and successful manipulation of NK cells *in vitro*. Furthermore, given how energy-intensive production of cytokines can be, cytokine secretion will be one of the centerpieces in the discussion of NK cell metabolism. Finally, some of these cytokine functions (especially immunosuppression) will be revisited in the analysis of the tumor milieu.

5. Role of NK Cells in Cancer Immunosurveillance

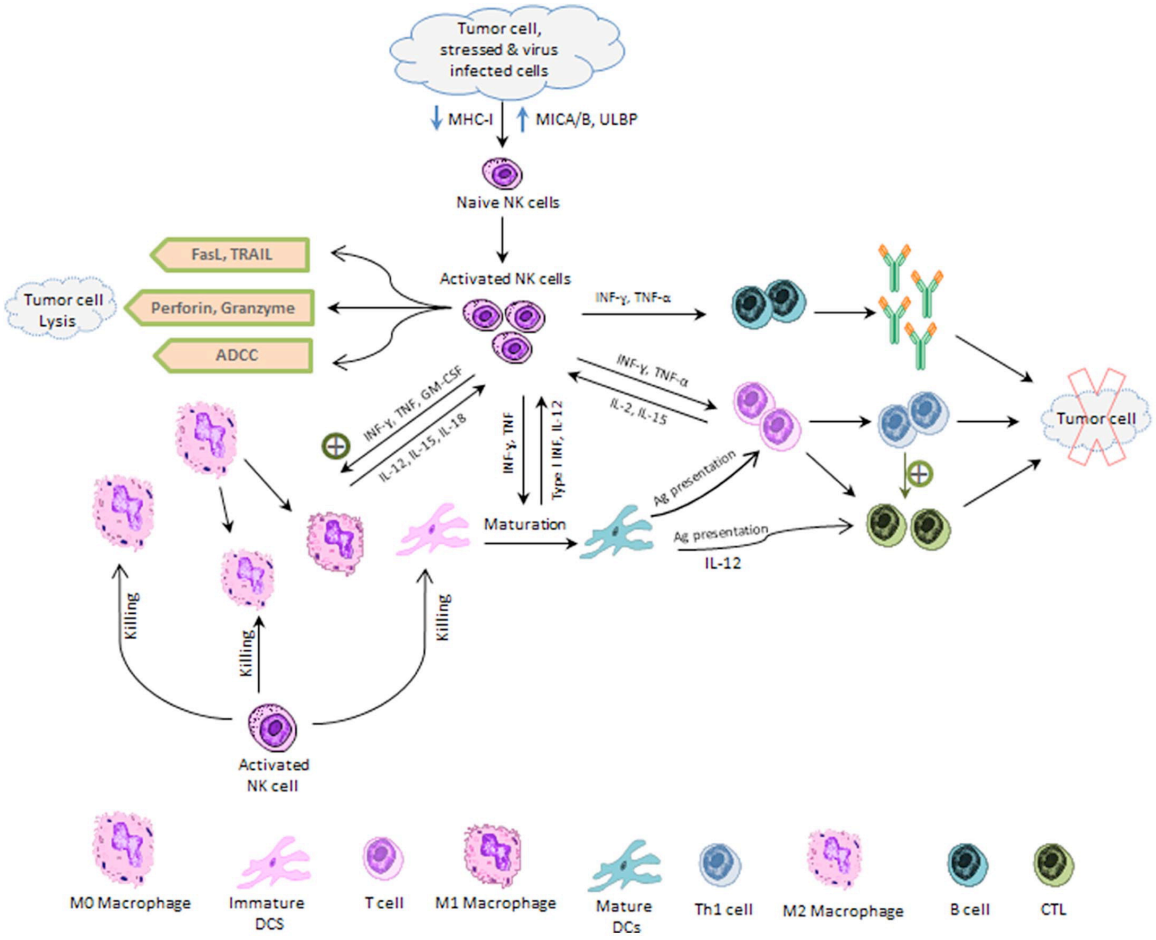

Figure 12. The Role of NK cells in Cancer Immunosurveillance. Combination of "missing self" and "stress-induced self" can activate NK cells which directly and indirectly mediate anti-tumor activity. Direct anti-tumor activity uses death receptor ligands (e.g. TRAIL, FasL), ADCC (via CD16), and cytotoxic degranulation. Activated NK cells secrete cytokines that induce maturation of various immune cell types, which in turn secrete pro-inflammatory cytokines that further activate NK cells. NK cells also kill immature DCs and M0/M2 macrophages, each of which have low MHC class I expression. Adapted from (Bodduluru et al., 2015).

Given that the motivation for this thesis concerns the enhancement of NK cell anti-tumor activity, it is worth briefly reviewing the evidence that NK cells are relevant to

cancer immunosurveillance. Even more, with the illustration of effector functions in NK cells, a coherent description of NK cell anti-tumor activity can be provided (Figure 12). For instance, evidence suggests that highly complex karyotypes (i.e. aneuploidy resulting from chromosome mis-segregation) lead to upregulated expression of various ligands for activating NK receptors, such as MICA/B, ULBP1/2, CD155, and CD112 (Santaguida et al., 2017). Aneuploidy is also associated with production of other pro-inflammatory signals, including IL-6, IL-8, CCL2, and the Senescence-Associated Secretory Phenotype (SASP) gene signature *in vitro* (Santaguida et al., 2017). These signals not only further activate NK cells, but can also activate other immune cell types, which ultimately secrete more inflammatory cytokines in a feed-forward loop (Bickel, 1993; Raulet & Guerra, 2009; Toshio Tanaka, Narazaki, & Kishimoto, 2014; Yoshimura, 2018). Given that at least 90% of solid tumors exhibit aneuploidy, this is likely important for NKG2D and/or DNAM-1 dependent clearance of tumor cells (Gordon, Resio, & Pellman, 2012; Holland & Cleveland, 2009; Santaguida et al., 2017).

The role of NK cells in cancer immunosurveillance should not be surprising, as NK cells were first identified in the setting of lysing a cell line (K562) that was derived from a leukemia patient (Ortaldo, Oldham, Cannon, & Herberman, 1977). Numerous early studies demonstrated the importance of NK cell activity in murine models (Stagg & Smyth, 2007). Mice deficient for NK cells through either genetic manipulation or antibody depletion exhibit impaired tumor suppression, which can be restored with adoptive transfer of NK cells or selective stimulation of remaining NK cells. Indeed, NK cell function in these murine models inhibits tumor initiation, progression, and metastasis

(Halfteck et al., 2009; S. Kim, Iizuka, Aguila, Weissman, & Yokoyama, 2000; Ksienzyk et al., 2011; M. J. Smyth, Cretney, et al., 2001; M. J. Smyth, Crowe, & Godfrey, 2001; Zhou, Kawakami, Higuchi, Yamashita, & Hashida, 2012).

With regard to NK cell cancer immunosurveillance in humans, a seminal, longitudinal study published in 2000 revealed that decreased NK cell activity correlated with increased risk of cancer incidence (Imai, Matsuyama, Miyake, Suga, & Nakachi, 2000). Similarly, decreased NK cell activity has been found in hereditary colorectal adenocarcinoma and metastatic melanoma (Jović, Konjević, Radulović, Jelić, & Spuzić, 2001; Markowitz et al., 1986; Warren, Stembridge, & Gardner, 1985). Of course, this does not necessarily exclude the explanation that decreased NK cell activity results from the immunosuppressive effects of advanced cancer. For instance, tumor growth has been shown to impair NK cell maturation in BM (Richards et al., 2006). Moreover, as this thesis will later discuss, it has been amply demonstrated that cancer cells suppress NK cell activity with secretion of numerous molecules including TGF-β, indoleamine 2,3-dioxygenase (IDO) , prostaglandin E2 (PGE2), vascular endothelial growth factor (VEGF), and adenosine (Baginska et al., 2013). In view of the evidence, is likely that NK cells prove critical to preventing tumor initiation. But once a tumor is established, mechanisms of immune evasion render the local and systemic immune system progressively dysfunctional, leading to more tumor growth in a feedback loop. However, the emerging success of immunotherapies—whether allogeneic adoptive NK cell therapy, cytokine infusion (Conlon et al., 2015; P. S. Kim et al., 2016; M. C. Ochoa et al., 2013), chimeric antigen receptor (CAR) NK cells (Altvater et al., 2009; Chu et al., 2014)

monoclonal antibodies (Gluck et al., 2004), or checkpoint inhibitors (S. Nguyen et al., 2009)—suggests that this cycle can be overcome. In fact, this thesis stipulates that manipulation of metabolism in NK cells may very well make each of these immunotherapies more effective. Even more, it may become a method of immunotherapy in its own right. Next, this section explicitly considers the basis of this thinking— the influence of intermediate metabolism on each stage in NK cell biology.

THE ROLE OF METABOLISM IN NK CELL BIOLOGY

From the previous discussion of NK cell biology, it is clear that NK cells must adopt a variety of functional profiles. NK cells need to be quiescent, rapidly inflammatory, or immunosuppressive depending on various stimuli in a broad range of environments. Mixed functional phenotypes add even more complexity. Given that metabolism facilitates function, it should be no surprise that the functional plasticity of NK cells demands a corresponding metabolic plasticity. This is not unique to NK cells. Virtually all immune cells exhibit flexibility in both functional and metabolic phenotypes (Mah & Cooper, 2016; P. J. Murray et al., 2015). In less than a decade, research into the metabolism of immune cells—so called immunometabolism—has revealed that metabolism can be both servant and master of immune function (Loftus & Finlay, 2016). This new paradigm has been built on the study of mostly T cells and macrophages and includes three major metabolic models divided along functional phenotype (Figure 13) (Mah & Cooper, 2016). However, as the complement to T cells and the bridge between innate and adaptive immunity, NK cells have received increasing interest (Mah & Cooper, 2016). This section will address that newfound interest and evaluate the role of metabolism in NK cell biology from development to effector function.

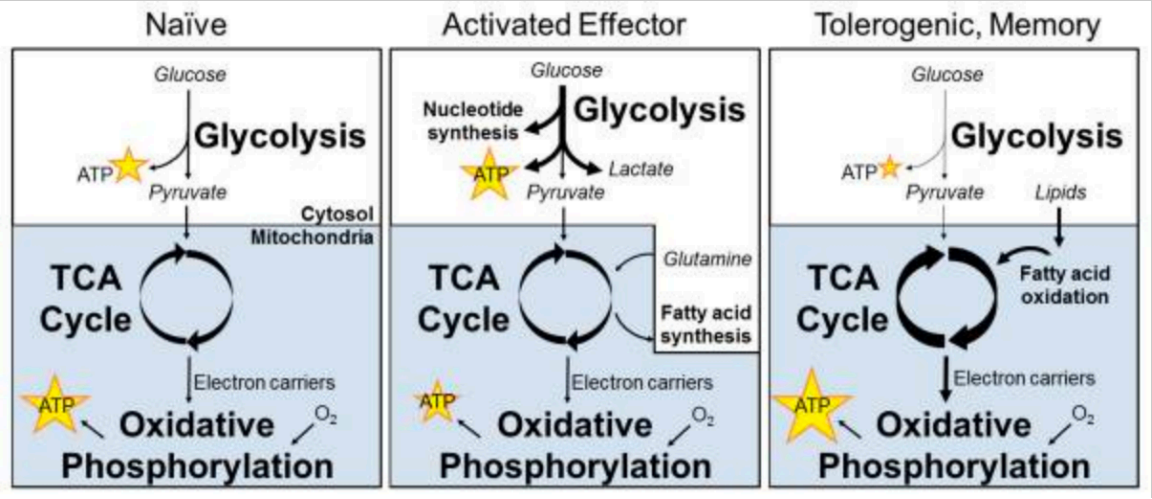

Figure 13. Paradigm of Immunometabolism. Left panel: naïve immune cells generally use glucose to fuel to ATP (adenosine triphosphate) and pyruvate production. Pyruvate is then further oxidized in the tricarboxylic acid (TCA) cycle. Nicotinamide adenine dinucleotide + hydrogen (NADH) and flavin adenine dinucleotide dihydrogen ($FADH_2$) bring electrons to the electron transport chain (ETC) to generate a proton gradient that leads to more ATP synthesis. Center panel: activated T cells, DCs, and M1 macrophages upregulate glycolysis so much so that cells generate substantial lactate. Meanwhile, intermediate metabolites are siphoned off as biosynthetic precursors (for nucleotide synthesis, fatty acid synthesis (FAS), etc.). Right panel: Tregs (regulatory CD4+ T cells), memory T cells and M2 macrophages rely more on oxidative phosphorylation (OXPHOS) fueled by fatty acids instead of glucose. Adapted from (Mah & Cooper, 2016).

A. Metabolism in NK Cell Development

Most data on the metabolism of NK cell development comes from murine models. While the developmental stages of NK cells in mice versus humans do not have exact parallels, the trends observed in mice may still be useful. Different stages of murine NK cell development are characterized by combinations of two surface antigens—TNF family member CD27 and the integrin CD11b. From most immature to mature, murine NK cells proceed as CD27+/CD11b- to CD27+/CD11b+ to CD27-/CD11b+ (Chiossone et al., 2009; Hayakawa & Smyth, 2006). In 2014, Marcais and colleagues monitored the

metabolic changes associated with the differentiation and maturation of murine NK cells. In BM, during the transition from CD27+/CD11b+ to CD27-/CD11b+, murine NK cells demonstrate two to threefold decreases in glucose uptake and expression of the nutrient transporters CD71 (transferrin receptor) and CD98 (heavy chain of amino acid transporter). There is also a coincident decrease in cell size and granularity with terminal differentiation (Marçais et al., 2014). Though splenic murine NK cells exhibit decreased expression of CD98 and CD71 compared to BM-resident NK cells at similar developmental stages, these peripheral NK cells also show trends of reduced nutrient transporter expression with maturation (Marçais et al., 2014). Gene signatures for different developmental states also differ. Mature NK cells upregulate gene expression of molecules in pathways related to quiescence such as autophagy, fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS), while more immature subsets upregulate expression of genes associated with cell growth (Marçais et al., 2014).

A key node thought to mediate these metabolic changes during NK cell development is mTOR (mechanistic target of rapamycin) (Poznanski et al., 2018). mTOR is a serine/threonine kinase that forms two distinct complexes, mTORC1 (mTOR complex 1) and mTORC2, defined by the components Raptor and Rictor, respectively (Yang et al., 2018). These complexes control several pathways crucial to cell proliferation and growth in multiple cell types. Through phosphorylation of 4EBP (binding protein of translation/initiation factor eIF4E) and S6 ribosomal kinase (S6K), mTORC1 can control translation. Moreover, mTORC1 is also known to control lipid synthesis through activation of sterol regulatory element binding protein (SREBP) and to

enhance glycolytic metabolism through promotion of transcription factors c-Myc and hypoxia-inducible factor 1α (HIF-1α) (Laplante & Sabatini, 2012; Marçais et al., 2014). Consistent with this, mTOR signaling and glycolytic metabolism progressively decrease during murine NK cell maturation. Furthermore, either *mtor* knockout or inhibition of mTORC1 with rapamycin correlates with reduced numbers of mature cells. Specifically, there is decreased transition from CD27+/CD11b- to CD27+/CD11b+ (Marçais et al., 2014). In view of this, mTOR signaling likely mediates metabolic changes in murine NK cells during maturation and differentiation. The role of mTOR in NK cell metabolism will be explored further in later sections.

B. Metabolism in NK Cell Activation

As observed in other immune cell types (e.g. M1 macrophages, CD4+ T cells, etc.) activation of NK cells usually induces significant changes in metabolism, especially glycolysis. This section will review important studies on the metabolic phenotypes of activated NK cells. Most experiments so far have been conducted with murine NK cells, while studies in human NK cells are more limited. Given the features unique to NK cells of mice and men, the material on each is presented separately.

1. Metabolism of Activated Murine NK Cells

The same group that explored NK cell metabolism and development was also one of the first to delve into the metabolism of NK cell activation. Specifically, Marçais et al. injected synthetic RNA duplex polyinosinic-polycytidylic acid (poly (I:C)) in mice and

later isolated the splenic NK cells. (Poly (I:C) simulates viral infection as a ligand of Toll-like receptor 3 (TLR3) and retinoic acid-inducible gene I (RIG-I). Both are pattern recognition receptors of innate immune system. Presumably, poly (I:C) promotes DC-mediated trans-presentation of IL-15). These NK cells exhibited increased expression of the nutrient transporters CD98 and CD71. In this study, glucose transporters such as GLUT1 were not observed, however glucose uptake increased as well. As in the developmental experiments, these responses were blunted in mTOR-deficient mice (whether mTOR-deficiency was induced genetically or chemically) (Marçais et al., 2014).

In vitro, Marçais et al. also examined the effects of direct low-dose (5 ng/mL) IL-15 stimulation on glycolysis and OXPHOS, using the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) as proxies, respectively (Figure 14). Murine NK cells had low basal rates of ECAR and OCR, but with IL-15 stimulation these metabolic parameters significantly increased. Moreover, the addition of oligomycin (OXPHOS inhibitor) had no effect on ECAR. Similarly, the addition of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (uncouples OXPHOS from ATP (adenosine triphosphate) synthesis) had no effect on OCR. Together, these inhibitors demonstrate that murine splenic NK cells were operating at maximal capacity with low-dose IL-15 stimulation (i.e. no glycolytic reserve or spare respiratory capacity) (Marçais et al., 2014).

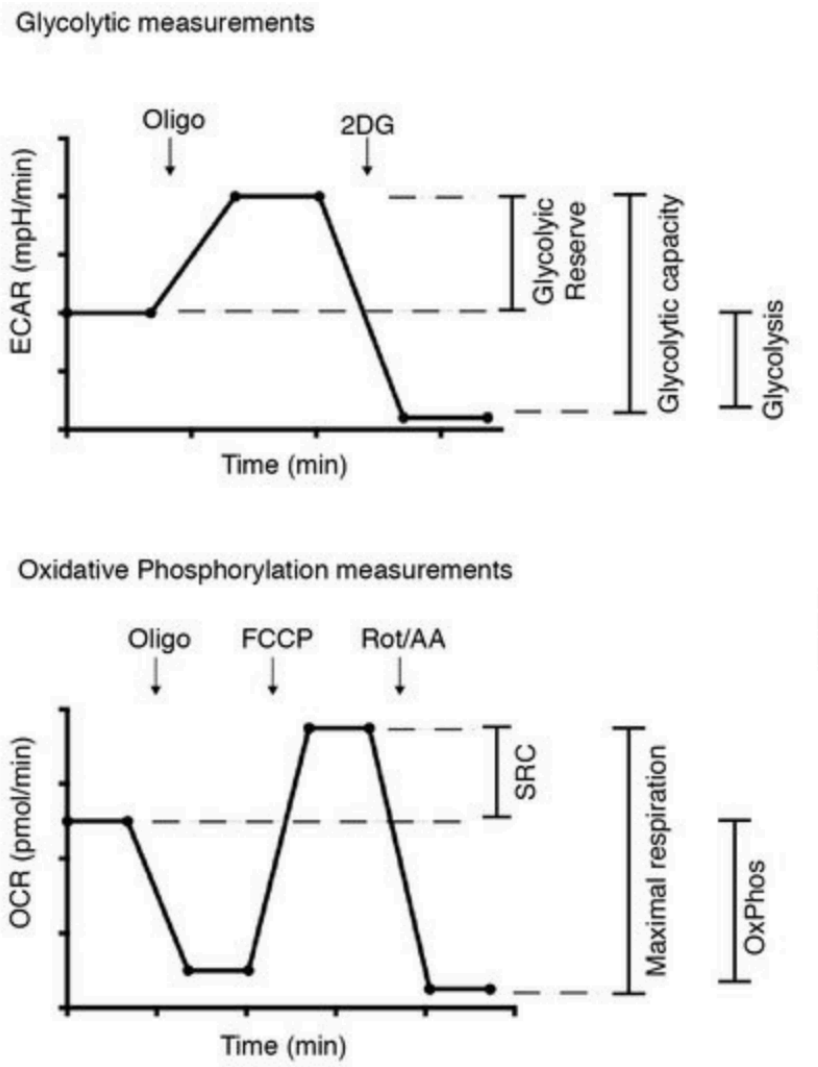

Figure 14. Measuring Central Carbon Metabolism. Top panel shows how to interpret graphs of ECAR to determine glycolytic reserve, basal rate of glycolysis, and glycolytic capacity using a Seahorse apparatus. Bottom panel present an interpretation of OCR to assessment spare respiratory capacity (SRC), maximal respiration, and basal rate of oxidative phosphorylation (OXPHOS). Note: Rot = Rotenone, a mitochondrial complex I inhibitor; Antimycin A = AA, a mitochondrial complex III inhibitor; FCCP = Carbon cyanide-4-(trifluoromethoxy)phenylhydrazone, uncouples oxidative phosphorylation from ATP production. Adapted from (Assmann et al., 2017).

Furthermore, in support of the importance of mTOR in NK cell metabolism, poly (I:C) and IL-15 stimulation were both shown to increase mTORC1 activity as measured by phosphorylation of known downstream targets (Marçais et al., 2014). Meanwhile, another group made the relationship between mTORC1 signaling activity and the metabolism of activated murine NK cells more explicit. Donnelly and colleagues demonstrated that mTORC1 signaling promoted expression of mRNA encoding glycolytic machinery such as lactate dehydrogenase A (LDHA), hexokinase 2 (HEX2), and GLUT1 in the context of poly (I:C) or IL-12 and IL-2 stimulation. In addition, consistent with the mRNA expression profile, Donnelly et al. showed that cytokine stimulation with IL-12 and IL-2 (synergistic given that IL-12 induces high affinity IL-2 receptor, no effect with IL-12 alone) significantly increased ECAR and OXPHOS. Furthermore, ECAR but not OXPHOS could be inhibited by rapamycin, edifying the role of mTORC1 in glycolysis (Donnelly et al., 2014).

The following year, Keppel et al. finally considered differences in cytokine versus receptor-mediated activation of NK cells. In an *in vitro* murine model, this group tested cytokine combinations IL-12 (10 ng/mL) /IL-15 (10 or 100 ng/mL), IL-12/IL-18 (50 ng/mL), or stimulated primary murine NK cells with antibodies against activating receptors NK1.1 and Ly49D (ITAM-bearing receptors unique to murine NK cells). In contrast to the aforementioned studies with activation regimes lasting from 18 hours to 5 days, this group considered short-term (4-6 hours) activation. No significant changes in either ECAR, OCR, or intracellular ATP production were observed over this timeframe. Under these conditions, OXPHOS appears to be glucose-driven given that oligomycin

and 2-deoxy-glucose (2DG, an early glucose inhibitor) reduce intracellular ATP production to the same levels while etomoxir (FAO inhibitor) had no effect (Keppel, Saucier, Mah, Vogel, & Cooper, 2015).

The most recent series of experiments have sought to explore the specifics of mTORC1 signaling required for NK cell activation and the associated metabolic phenotype. One group investigated SREBP—a target of mTORC1 signaling which controls lipid synthesis. Not only do murine NK cells upregulate glycolysis upon activation, these cells also increase in size (i.e. blastogenesis). Considering the required membrane components for this increase in size, it would be reasonable to think that lipid synthesis—and therefore SREBP—could be essential to the metabolic phenotype of activated NK cells.

In 2017, Assmann et al. demonstrated that SREBP is critical, but not in the way that was expected. With 18 hours of low to moderate doses of IL-2 (20 ng/mL) and IL-12 (10 ng/mL), NK cells exhibit increased glycolysis, glycolytic capacity, OXPHOS, and respiratory capacity. Furthermore, metabolomic tracing experiments revealed that glucose fueled amino acid and fatty acid synthesis. Unsurprisingly, mRNA expression of SREBP transcription factors and the associated target genes to support *de novo* lipid synthesis were also upregulated (e.g. fatty acid synthase (*Fasn*), stearoyl-coenzyme A (CoA) desaturase 1 (*Scd1*)) and cholesterol synthesis (e.g. 3-hydroxy-3-methylglutaryl-CoA synthase 1 (*Hmgcs1*) and acetyl-CoA acetyltransferase 2 (*Acat2*)). (Assmann et al., 2017).

However, while direct chemical inhibition of mTORC1 and SREBP inhibited these metabolic changes in response to IL-2/IL-12 cytokine stimulation, inhibition of *de novo* lipid synthesis had minimal effects. Meanwhile, two other targets of SREBP—the components of the citrate-malate transporter in mitochondria—did have major effects on murine NK cell metabolism. ATP-citrate lyase (ACLY) and SLC25A1 together form the citrate-malate antiporter. SLC25A1 exports citrate from the mitochondria while simultaneously importing malate. ACLY splits the exported citrate into oxaloacetate (OAA) and Acetyl-CoA. Acetyl-CoA can participate in further lipid synthesis reactions while OAA reduction in the cytosol produces malate and regenerates NAD+ (nicotinamide adenine dinucleotide) from NADH (NAD and hydrogen) (Figure 15) (Assmann et al., 2017). In this way, malate can return the mitochondria via SLC25A1 to complete the cycle. Effectively, the citrate-malate shuttle can regenerate cytosolic NAD+ while fueling OXPHOS with NADH. Under conditions of IL-2/IL-12 stimulation, murine NK cells seem to rely almost exclusively on the citrate-malate shuttle to support OXPHOS. Indeed, the rest of tricarboxylic acid (TCA) cycle is essentially irrelevant to OXPHOS. Moreover, ACLY inhibition substantially increases lactate production (using up glycolytic reserve) as the NK cell compensates for significantly compromised OXPHOS activity. Though the rate of glycolysis increases, it is worth mentioning that ACLY inhibition does reduce maximum glycolytic capacity (Assmann et al., 2017).

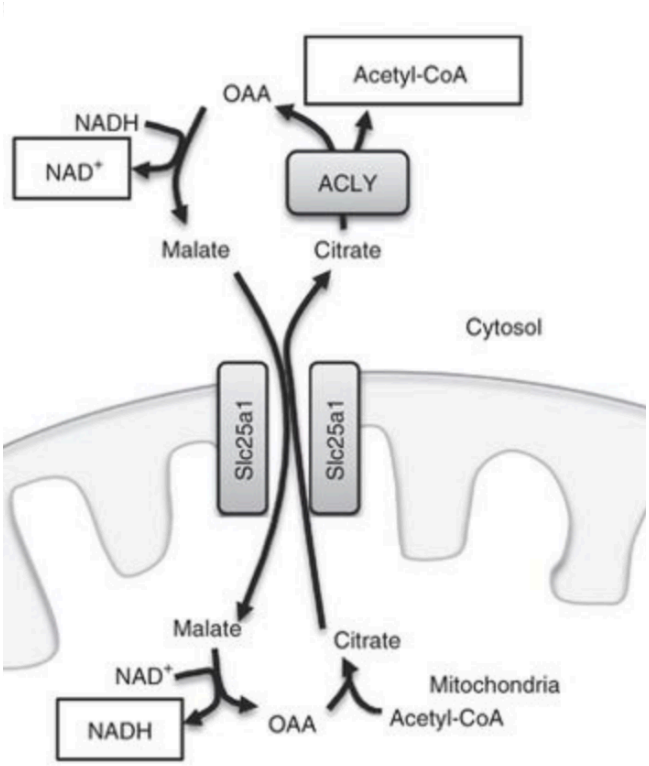

Figure 15. Citrate-Malate Shuttle. Citrate generated from pyruvate in mitochondria can be exported via the citrate-malate antiporter SLC25A1, after which it is split into acetyl-CoA and OAA. OAA is reduced to malate, which is exchanged for citrate, thereby completing the cycle. Adapted from (Assmann et al., 2017).

Another known target of mTORC1—cMyc—also increases expression in murine NK cells upon 18-hour stimulation by IL-2 and IL-12. *Myc* knockout renders murine NK cells unable to increase in size, upregulate metabolism (e.g. glycolysis, glycolytic capacity, OXPHOS, respiratory capacity), or increase nutrient receptor expression (e.g. CD71 and CD98) in response to IL-2/IL-12 stimulation. (Loftus et al., 2018). Furthermore, these *Myc* knockout mice exhibited decreased mitochondrial mass and

decreased mRNA expression of glycolytic machinery such as *Ldha*, *Glut1*, *Hex2,* and the pyruvate kinase isozymes M1 and M2 (*Pkm1* and *Pkm2*). (Recall that the first three mRNAs are associated with increased mTORC1 activity) (Donnelly et al., 2014; Loftus et al., 2018). Through a series of elegant inhibitor experiments, Loftus et al. demonstrated that cMyc expression and mTORC1 signaling is dependent on amino acid uptake through the heterodimeric amino acid transporter comprised of CD98 and SLC7A5. Though cMyc may be a downstream target of mTORC1, surprisingly, the dependence on mTORC1 signaling is removed with long-term cytokine stimulation. Moreover, just like the citrate-malate shuttle, SLC7A5 is an obligate antiporter, usually exchanging intracellular glutamine for extracellular amino acids. As such, culture in glutamine-free media reduced cell size, CD71 expression and all metabolic parameters associated with glycolysis and OXPHOS. A similar metabolic defect manifests in the presence of an SLC7A5 inhibitor. This SLC7A5 dependence exists because cMyc is aggressively targeted for degradation in the murine NK cell. In fact, with inhibition of either proteasomes or glycogen synthase kinase 3 (GSK3) (which facilitates ubiquitination of cMyc), one can rescue cMyc expression under glutamine withdrawal or SLC7A5 inhibition (Loftus et al., 2018).

In addition, to assess the influence of glutamine anaplerosis (wherein glutamine fuels the TCA cycle after conversion to α-ketoglutarate), Loftus et al. observed the effects of a glutaminase inhibitor on NK cell metabolism. Effects on metabolic parameters, particularly OXPHOS, were minimal. Indeed, glutamine need only be present to support cMyc synthesis, not to fuel OXPHOS (Loftus et al., 2018).

Lastly, Altamutairi et al. examined the specific effects of IL-18 on murine NK cell metabolism. All experiments were conducted with baseline low dose (~2 ng/mL) of IL-2 to enhance cell survival. In a dose-dependent fashion (from 0 to 100 ng/mL), IL-18 was shown to induce upregulation of nutrient transporters (especially CD98 and CD71) and glucose uptake. Moreover, IL-12 or IL-15 synergized with IL-18 to increase nutrient transporter expression, but did not vary in a dose-dependent fashion. In contrast, IL-12 or IL-15 alone had no effect on metabolism. IL-18 had such a powerful effect on metabolism that it could rescue NK cells from rapamycin inhibition. Finally, IL-18 could even activate mTORC1 by upregulating amino acid uptake, specifically by leucine and glutamine exchange through increased expression of the heterodimer CD98/SLC7A5 (Almutairi et al., 2019).

In summary, murine NK cells tend to upregulate to nutrient receptors, glycolysis, and OXPHOS in response to long-term (>18 hours) exposure to various cytokine cocktails including IL-2, IL-12, IL-15, and IL-18 (Donnelly et al., 2014; Marçais et al., 2014). Under these conditions, OXPHOS is primarily fueled by glucose—neither fatty acids nor glutamine (Assmann et al., 2017; Donnelly et al., 2014; Loftus et al., 2018). Two semi-independent nodes of control—SREBP and cMyc—exist downstream of mTORC1, a known regulator of glycolytic metabolism (Figure 16). (O'Brien & Finlay, 2019). (Notably, knockout of the mTORC1 target HIF-1α does not affect NK cell metabolism with IL-2/IL-12 stimulation under normoxic (~20% O_2) conditions) (Loftus et al., 2018). SREBP exerts control through the citrate-malate transporter to support OXPHOS and glycolysis with cytosolic NAD+ and/or mitochondrial NADH (Assmann et

al., 2017). cMyc exerts control through transcriptional upregulation of mRNA for various enzymes of the glycolytic machinery and promotes mitochondrial biogenesis (Loftus et al., 2018). The next section will demonstrate that similar metabolic phenotypes are observed in certain subsets of human NK cells.

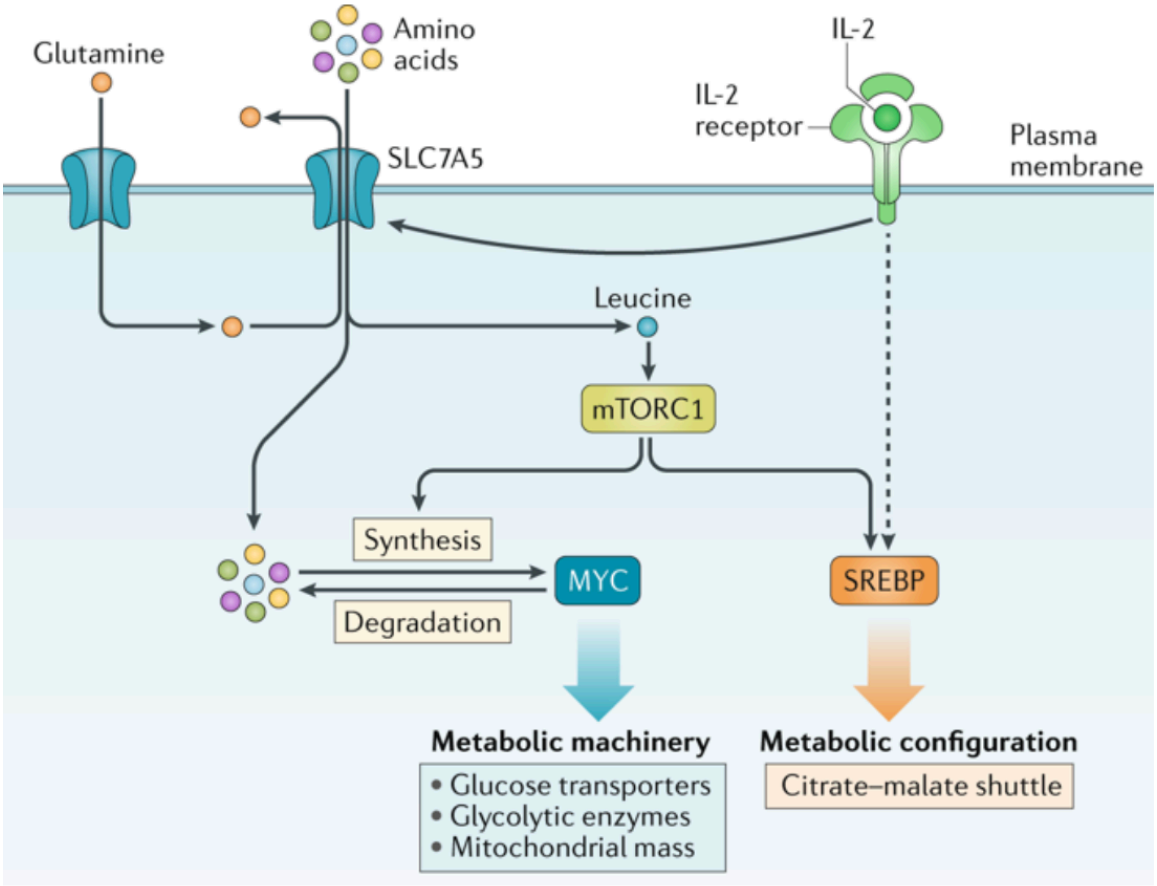

Figure 16. Two Major Nodes of Murine NK Cell Metabolism. Transcription factors MYC and SREBP control the metabolic configuration of activated murine NK cells. mTORC1 is an upstream regulator of both these nodes. SREBP promotes the citrate-malate shuttle while cMyc upregulates various glycolytic enzymes, increases expression of nutrient transporters, and stimulates mitochondrial biogenesis. Intracellular glutamine allows import of various amino acids (in particular leucine) which supports cMyc synthesis, overcoming the high rate of cMyc degradation. Adapted from (O'Brien & Finlay, 2019).

2. Metabolism of Activated Human NK Cells

While investigations into the metabolism of murine NK cells made no attempts to distinguish the metabolic phenotypes of different NK cell subsets, human NK cell studies explicitly considered possible differences. One of the early studies divided human NK cells according to CD56 expression with the classic designation of $CD56^{bright}$ vs $CD56^{dim}$. These NK cells were isolated from PBMCs of healthy donors and therefore qualify as members of the cNK subset (Keating et al., 2016). A later study considered not only cNK, but also trNK cells of the liver and spleen isolated from patients undergoing liver transplants or abdominal tumor excision surgeries (Salzberger et al., 2018).

Perhaps unsurprisingly, given the different sources of NK cells, the aforementioned papers disagree on which CD56 subset displays greater nutrient receptor expression (e.g. GLUT1, CD98, and CD71). Keating et al. paint the PB $CD56^{bright}$ NK cell as a metabolic powerhouse with not only greater baseline glucose uptake and nutrient receptor expression compared to PB $CD56^{dim}$, but also more dramatic increases in glucose uptake, glycolytic metabolism, and OXPHOS in response to low-dose IL-2 (~10 ng/mL) or moderate to high dose IL-12 (30 ng/mL) and IL-15 (100 ng/mL) 18-hour stimulation. The IL-12/IL-15 combination produced the most profound effects. Conspicuously, while all nutrient transporters increased with cytokine activation (by either regimen) GLUT1 increase was minimal in PB $CD56^{bright}$ NK cells, despite the significant increase in glucose uptake (Keating et al., 2016). This suggests that either GLUT1 wasn't operating at top capacity until glucose was used more readily in glycolysis or there are other glucose transporters (e.g. GLUT3, GLUT4, GLUT8) that the

PB CD56^{bright} NK cell subset preferentially upregulates under these cytokine activation conditions. Moreover, rapamycin inhibited IL-2 induced increases in CD71 and CD98 expression along with the upregulation in glycolytic metabolism. In contrast, IL-12/IL-15 induced glycolytic metabolism was mTORC1-independent. The same was true of OXPHOS for either cytokine activation regimen (Keating et al., 2016).

On the other hand, Salzberger et al. present more nuanced nutrient receptor data. In their study, PB CD56^{dim} cells displayed greater GLUT1 expression yet lower expression for CD71 and CD98 relative to PB CD56^{bright} cells. This relationship holds for trNK cells in the liver and spleen as well as a third subset the researchers define—tissue-derived NK cells (tdNK). These NK cells lack the phenotypic markers of tissue-residence yet still occupy the tissues. Among these disparate subsets, PB NK cells demonstrate relatively greater GLUT1 expression, trNK cells exhibit greater CD71 and CD98 expression, and tdNK cells display a nutrient expression profile intermediate to the other subsets yet skewed to trNK. With low dose IL-12 (5 ng/mL) and IL-15 (2.5 ng/mL) all subsets increase nutrient transporter expression. Specifically, the CD56^{bright} subsets PB cells upregulate GLUT1 much greater than trNK or tdNK and unexpectedly, trNK upregulates GLUT1 greater than tdNK (Salzberger et al., 2018).

Stepping back from the CD56 classification, Veláquez et al. uniquely consider human cNK cell metabolism under conditions of short-term hypoxia (1% O_2, 28 hours) and moderate dose IL-15 (45 ng/mL) priming (6 hours). The human cNKs were isolated from the PBMCs of healthy donors. Regardless of oxygen tension, IL-15 priming tended to upregulate energy production and anabolism. Specifically, with priming, hypoxia

synergistically induced transcriptional upregulation of genes associated with HIF-1α signaling and glycolysis/gluconeogenesis. This corresponded with greater rates of glycolysis, glycolytic capacity, and glycolytic reserve. Even in normoxic conditions, IL-15 primed NK cells still exhibited dependence on HIF-1 for increased glycolytic activity. Inhibition of HIF-1 decreased glycolytic activity and viability of NK cells regardless of oxygen tension. (Velásquez et al., 2016).

Though IL-15 priming seems to provide metabolic advantages to human NK cells under conditions of hypoxia, long-term IL-15 exposure (on the order of a week or more) may lead to metabolic defects in human NK cells. In 2018, Felices et al. devised an IL-15 priming regimen of continuous IL-15 at 10 ng/mL for 9 days (IL-15cont) versus a protocol in which there was a 3-day "gap" in IL-15 exposure from days 4-6 (IL-15gap). The IL-15cont group of NK cells had similar glycolytic parameters as measured by ECAR, but displayed defects in OXPHOS compared to the IL-15gap cohort. Indeed, the IL-15cont NK cells had significantly reduced spare respiratory capacity. Etomoxir—a carnitine-palmitoyl transferase I (CPT1) inhibitor—did not affect IL-15cont NK cells, but removed the additional spare respiratory capacity exhibited in IL-15gap NK cells. This suggests that continuous, long-term IL-15 stimulation may induce dysfunctional FAO (mediated by CPT1). Interestingly, mTORC1 inhibition by rapamycin could recover some spare respiratory capacity in IL-15cont NK cells, though glycolytic metabolism decreased in response (Felices et al., 2018).

Another distinction between NK cells worth exploring concerns "educated" vs "uneducated" NK cells. Recall from a previous section that educated NK cells more

vigorously respond to stimulation and demonstrate improved cytotoxicity relative to uneducated NK cells. In 2018, Pfeifer et al. tried to sort out any metabolic differences that might correspond with "education" in human PB NK cells isolated from the PBMCs of several healthy donors. These cells were subject to low dose (5 ng/mL) IL-15 overnight expansion before target cell activation. Target cell activation of NK cells involved exposure to the tumor cell lines K562 or 722.221 *in vitro.* (Pfeifer et al., 2018).

At baseline, educated NK cells exhibited greater GLUT1 expression and glucose uptake relative to uneducated counterparts. These parameters increased with target cell activation for both educated and uneducated NK cell subsets. Moreover, while educated NK cells had higher rates of glycolysis, other glycolytic parameters (e.g. glycolytic capacity, glycolytic reserve) as well as OXPHOS were similar between educated and uneducated NK cell subsets (Pfeifer et al., 2018). Researchers then analyzed the results after stratifying the educated subset into groups based on the primary self-recognizing inhibitory receptor—NKG2A or KIR. KIR+ educated NK cells had the highest GLUT1 expression of any subset, while NKG2A+ educated NK cells had GLUT1 expression similar to uneducated NK cells. Even with less GLUT1 expression, NKG2A+ had greater glucose uptake than KIR+ educated NK cells, which in turn had greater glucose uptake than uneducated NK cells. Much like the PB $CD56^{bright}$ NK cells earlier, this disparity might be explained if NKG2A+ educated NK cells express other glucose transporters besides GLUT1 (Pfeifer et al., 2018).

In the same year, Schafer et al. focused on KIR+ educated NK cells isolated from human PBMCs. Cells were expanded for a much longer period (14-21 days) using K562

Clone9.mbIL21 feeder cells (which induce proliferation of NK cells with membrane-bound IL-21) or just freshly isolated. Freshly isolated NK cells had no differences in glucose uptake, glycolytic metabolism, or OXPHOS between KIR+ educated and uneducated subsets (Schafer et al., 2019). However, expanded KIR+ educated NK cells had significantly greater rates of glycolysis, glycolytic capacity, and glycolytic reserve compared to uneducated NK cells. In these same subsets GLUT1 and GLUT3 expression did not differ, although GLUT8 expression was slightly greater in the KIR+ educated subset. Unfortunately, the researchers leave readers to wonder if expanded NK cells exhibit differences in glucose uptake. That would answer the question of whether glycolytic metabolism in KIR+ educated NK cells versus uneducated NK cells is independent of the glucose transporter expression profile under these conditions (Schafer et al., 2019). Meanwhile, KIR+ educated NK cells were shown to have different levels of certain metabolites relative to uneducated NK cells. KIR+ educated NK cells had significantly lower levels of glutamate, aspartate, and taurine with trends towards increased lactate and acetate versus uneducated cells (Schafer et al., 2019).

Taking a "shot-gun" proteomics approach, Schafer et al. determine that KIR+ educated NK cells express more of proteins involved in cell metabolism such as macrophage migratory inhibitory factor (MIF, a glycosylation inhibitor), mitochondrial cytochrome c oxidase (MTCO2, last enzyme in the electron transport chain (ETC)), succinate dehydrogenase (SDHB), and PKM2. Similarly, with a phospho-proteomics approach, KIR+ educated NK cells have decreased ATM (ataxia telangiectasia mutated protein) phosphorylation when activated by NKp46 cross-linking and increased p38 and

adenosine monophosphate (AMP)-activated protein kinase α (AMPK-α) phosphorylation with KIR cross-linking relative to uneducated NK cells. It is unclear if/how these differences in protein expression contribute to divergent metabolic phenotypes (Schafer et al., 2019).

One final category that deserves attention is "adaptive" or "memory-like" NK cells. In 2018, Cichocki et al. identified the metabolic features of an NK subset that readily expands and persists in response to human CMV (HCMV) infection. These cells exhibit a bioenergetic profile similar to memory CD8+ T cells with increased OXPHOS, greater mitochondrial mass, and higher spare respiratory capacity (Cichocki et al., 2018; van der Windt et al., 2012, 2013). Furthermore, the metabolic adaptations of adaptive NK cells have been connected to the increased expression of the transcription factor AT-rich interaction domain 5B (ARID5B), which upregulates the ETC component ubiquinone-cytochrome c reductase binding protein (UQCRB) and the anti-apoptotic protein Bcl-2. In fact, in NK-92 cells (with IL-12/IL-18 stimulation), overexpression of ARID5B enhances mitochondrial fitness (as measured by mitochondrial membrane potential, OXPHOS, and spare respiratory capacity) while ARID5B knockout establishes a metabolic phenotype more consistent with "naïve" NK cells (Cichocki et al., 2018).

Because of the varying activating conditions, inferring a coherent model of NK cell metabolism in humans proves challenging. Two of the most glaring contradictions involve GLUT1 expression. While CD98 and CD71 show higher expression in $CD56^{bright}$ NK cells in PB and tissues compared to $CD56^{dim}$ counterparts, comparative GLUT1 expression varies according to the study. What is consistent about the GLUT1 expression

is that whichever subset has greater initial expression has less upregulation with cytokine stimulation (Keating et al., 2016; Salzberger et al., 2018). The second contradiction regards NK cell education. KIR+ educated cells either have increased or the same GLUT1 expression as uneducated cells. However, at least it can be agreed that educated cells likely have greater glucose uptake with cytokine stimulation (Pfeifer et al., 2018; Schafer et al., 2019).

NK cells may very well display myriad metabolic phenotypes depending on circumstances such as the host condition, identity and dose of cytokines, duration of stimulation, and even continuity of stimulation. In fact, IL-15 priming presents a convenient case study in the effect of the aforementioned variables on human NK cell metabolism. Short-term (6 hours) IL-15 exposure at a moderately high dose (45 ng/mL) synergizes with hypoxia to upregulate glycolytic parameters at greater levels than normoxia. These IL-15 priming conditions produce no measurable effects in OXPHOS (Velásquez et al., 2016). In contrast, continuous low-dose (though supraphysiologic) IL-15 priming at 10 ng/mL for nine days can induce defective OXPHOS as represented by reduced respiratory capacity. Meanwhile, another condition—intermittent IL-15 priming— avoids this metabolic defect entirely (Felices et al., 2018). Even more, while a high-dose IL-12 (30 ng/mL) + IL-15 (100 ng/mL) combination over 18 hours induces robust, mTORC1-independent glycolytic metabolism, the glycolysis in IL-15cont NK cells is vulnerable to mTORC1 inhibition (Felices et al., 2018; Keating et al., 2016). It is clear that researchers need to carefully understand the effects of different activation conditions before they can precisely exploit NK cell metabolism.

C. Metabolism in NK Cell Effector Function

Now that this thesis has already discussed the intersection of NK cell activation and metabolism, this section will discuss the other half—the intersection of metabolism and NK cell effector functions. Many of the same studies referenced earlier will be revisited. Here, methods to manipulate effector function—either directly or indirectly—will become apparent.

1. Metabolism of Murine NK Cell Effector Functions

In vitro, with poly(I:C) or IL-2/IL-12 stimulation, NK cell IFN-γ production and granzyme B expression require increased glycolytic flux and mTORC1 signaling activity. However, this dependence does not extend to the production other cytokines, such as TNF-α (Donnelly et al., 2014). Murine NK cells remain competent in TNF-α production despite rapamycin or 2DG treatment. Moreover, *in vivo* experiments provide similar evidence for the glycolytic dependence of IFN-γ production, though results differ somewhat in that granzyme B expression is unaffected by systemic rapamycin or 2DG treatment (Donnelly et al., 2014).

Cytokine stimulation may also lead to IFN-γ production that is independent of glycolysis. In fact, IL-12/IL-18 stimulation renders IFN-γ production by murine NK cells insensitive to inhibition of either glycolysis or OXPHOS. In contrast, IL-12/IL-15 mediated and receptor-mediated (e.g. NK1.1, Ly49D) IFN-γ production still depend on OXPHOS and glucose (Keppel et al., 2015). These differences can be partially explained

by regulation of the mRNA expression for *Ifng*. While IL-12/IL-18 stimulation quickly induces increased *Ifng* mRNA expression (though delayed 4-6 hours by oligomycin), receptor-mediated activation does not significantly change *Ifng* mRNA expression. Though the details of *Ifng* regulation are not clear, this implies that IL-12/IL-18 stimulation regulates *Ifng* mRNA at the level of transcription while activating receptors regulate *Ifng* mRNA post-transcription (Keppel et al., 2015). The latter pathway may very well need a metabolism-derived second signal. This requirement is not immutable though, since long-term (>72 hours), high dose (100 ng/mL) IL-15 priming can enable IFN-γ production of receptor-activated NK cells to become essentially metabolism-independent (i.e. OXPHOS-independent and drastically reduced glucose dependence) (Keppel et al., 2015). This may not actually confer any benefits in cytotoxicity, however. Mah et al. demonstrated *in vitro* that NK cells with long-term IL-15 priming exhibit variable cytotoxicity in the presence of the glycolysis inhibitor 2DG. More explicitly, 2DG treatment reduced Ly49H-mediated cytotoxicity against Ba/F3-m157 (MCMV-infected) targets, but had no effect on NKG2D-mediated cytotoxicity against YAC-1 tumor cells. Upon closer inspection, NK cells with 2DG treatment had decreased granzyme B expression and defective actin organization at the immunological synapse (Mah et al., 2017). *In vivo* murine models of MCMV-infection treated with 2DG or rapamycin also displayed reduced cytotoxicity, but unexpectedly had no changes in granzyme B expression or degranulation. *In vivo* cytotoxicity could be restored with treatment by the IL-15 superagonist ALT-803 though (Mah et al., 2017).

The two metabolic nodes downstream of mTOR mentioned in the previous section—SREBP and cMyc—are also relevant to NK cell effector functions (Assmann et al., 2017; Loftus et al., 2018; O'Brien & Finlay, 2019). *In vitro* and *in vivo,* SREBP inhibition by 25-hydroxycholesterol (25-HC, a metabolite of cholesterol) or PF429242 significantly decreases NK cell IFN-γ production, granzyme B expression, and cytotoxicity. Importantly, ACLY (a target of SREBP) inhibition mirrors the effects of direct SREBP inhibition on NK cell effector functions while inhibition of lipid synthesis (another target of SREBP) does not. Meanwhile, decreasing cMyc expression leads to many of the same effects as SREBP such as decreased IFN-γ production, reduced granzyme B expression, and impaired cytotoxicity. The important result of the series of cMyc experiments is that long-term glutamine withdrawal negatively affects NK cell effector function, but glutaminase inhibition does not. This implies that while glutamine anaplerosis is irrelevant to effector function, glutamine availability proves essential. Intriguingly, the triviality of glutaminase in NK cells contrasts significantly with T cells and several cancers (Loftus et al., 2018).

2. Metabolism of Human NK Cell Effector Functions

In what is now a recurring pattern, activation conditions influence metabolic dependence in human NK cell effector functions as well. In general, the NK cells most impervious to metabolic derangements belong to the "educated" and $CD56^{bright}$ subsets, though there is some variability. With low-dose, overnight IL-15 stimulation KIR-educated and uneducated NK cells exhibit decreased degranulation (as measured by CD107a expression) upon glucose deprivation. 2DG treatment magnifies this effect. Yet,

NKG2A-educated NK cells have no such handicap in glucose-free media (Pfeifer et al., 2018).

With more prolonged activation (2-3 weeks) by membrane-bound IL-21, neither glucose deprivation nor OXPHOS inhibition affects KIR-educated NK cell cytotoxicity against tumor cell line 722.221. Indeed, it takes aggressive inhibition with 2DG, shikonin (PKM2 inhibitor), glucose free media, and oligomycin to affect the cytotoxicity of the KIR-educated subset after feeder cell expansion. In contrast, uneducated NK cell cytotoxicity decreased by 87% in only the presence of oligomycin (Schafer et al., 2019).

IL-2 stimulated human NK cell effector functions vary in metabolic sensitivity. While IL-2 induced IFN-γ production and granzyme B expression is minimally affected by oligomycin, IL-2 stimulated NK cells have limited degranulation in the presence of the same inhibitor. In addition, direct inhibition of glycolysis in IL-2 stimulated NK cells has little effect on effector functions, however rapamycin treatment does reduce IFN-γ synthesis (Keating et al., 2016).

IL-12/IL-15 stimulated human NK cells are somewhat less robust in the face of metabolic inhibition. With this cytokine cocktail, inhibition of OXPHOS and/or glycolysis reduces IFN-γ synthesis. On the other hand, granzyme B is expression is relatively independent of OXPHOS and glycolysis (Keating et al., 2016). The results are more uniform in the context of SREBP inhibition. Inhibition of either SREBP or ACLY (SREBP target) significantly reduces granzyme B expression and IFN-γ production, especially for $CD56^{bright}$ NK cells (Assmann et al., 2017). The metabolic sensitivity of NK cell degranulation splits along the lines of CD56 expression. Though both CD56

subsets degranulate effectively regardless of glycolytic flux, only $CD56^{bright}$ NK cells degranulate well in the presence of oligomycin. Indeed, $CD56^{dim}$ NK cells have limited degranulation during OXPHOS inhibition (Keating et al., 2016).

Even without IL-12 supplementation, short-term (<24 hours) IL-15 priming can produce an interesting functional phenotype in NK cells. Under short-term hypoxic conditions (1%, 6 hours) human NK cells exhibit both increased migratory behavior and greater secretion of the chemokines CCL3, CCL4, and CCL5. Against K562, short-term hypoxia doesn't seem to affect target cell lysis compared to normoxic conditions. However, IL-15 priming and hypoxia do synergize to render NK cells more capable of inducing late stage apoptosis in target cells (Velásquez et al., 2016). With very long-term (>1 week), continuous IL-15 priming, NK cell effector function (e.g. decreased IFN-γ production and CD16 expression) can be compromised, yet this can be avoided by adopting a stimulation regimen with intermittent IL-15 exposure (Felices et al., 2018).

In the context of IL-12/IL-18 stimulation, recall that increased expression of the ARID5B transcription factor is a hallmark of "adaptive" NK cells that corresponds with greater increased respiratory capacity and mitochondrial mass. Apparently, NK-92 cells with greater ARID5B expression display increased IFN-γ synthesis while ARID5B knockout achieves the opposite. Surprisingly, ARID5B knockout has no effect on TNF secretion or degranulation (Cichocki et al., 2018).

In an earlier section, data on murine NK cells suggested that lipid synthesis might be less important than central carbon pathways to NK cell cytotoxicity. Specifically, inhibition of acetyl-CoA carboxylase (ACC, the first enzyme in fatty acid and

mevalonate synthesis) with TOFA (5-(tetradecyloxy)-2-furoic acid) did not negatively influence the cytotoxicity of murine NK cells against YAC-1 cells or the upregulation of metabolism observed with cytokine activation (Assmann et al., 2017). However, *in vitro* chemical inhibition of HMG-CoA reductase (a rate-limiting enzyme in mevalonate/cholesterol synthesis) via statins (especially lipophilic statins such as simvastatin and fluvastatin) has been shown to decrease cytotoxicity in human NK cells (Hillyard et al., 2007; Poggi, Boero, Musso, & Zocchi, 2013; Raemer, Kohl, & Watzl, 2009; Toru Tanaka, Porter, Horvath-Arcidiacono, & Bloom, 2007). Unfortunately, the results of investigations into the role of mevalonate metabolism on human NK cell function have been inconsistent. There is no consensus on whether mevalonate synthesis itself supports NK cell cytotoxicity or whether a downstream product such as cholesterol or geranylgeranyl pyrophosphate (GG-PP) is more important. Furthermore, while one study found that *in vitro* NK cell cytotoxicity trended (positively) with plasma cholesterol concentration regardless of a patient's previous statin use, cholesterol does not restore function in NK cells following *in vitro* statin exposure (Hillyard et al., 2007; Raemer et al., 2009). However, exogenous addition of mevalonate or GG-PP does reverse statin effects. The importance of isoprenoids (e.g. farnesyl pyrophosphate or GG-PP) is thought to be related to prenylation reactions. Prenylation using isoprenoid substrates can anchor small GTPases such Rho A and Rac1 to membrane rafts (i.e. microdomains) and support signaling of these associated molecules. In at least one study though, prenyl transferase inhibitors failed to replicate the effects of statin treatment (Raemer et al., 2009).

In most of these studies, granule exocytosis (i.e. degranulation) as measured by CD107a expression tends to be reduced in the presence of statins (Hillyard et al., 2007; Poggi et al., 2013; Raemer et al., 2009; Toru Tanaka et al., 2007). Evidence suggests that the machinery of exocytosis may not be affected, but instead early signaling associated with LFA-1 mediated conjugation (Poggi et al., 2013; Raemer et al., 2009). Furthermore, there may be a statin dose-dependent inhibition of ganglioside M1 membrane rafts relevant to aggregation of activating receptors at the immunological synapse (Hillyard et al., 2007; Poggi et al., 2013; Raemer et al., 2009; Toru Tanaka et al., 2007). Even more, statin effects may be activation-dependent. While NK cell cytotoxicity (through granzyme B/perforin release) mediated by engagement of DNAM-1, NKG2D, 2B4 and NCRs is significantly inhibited by statin exposure, CD16 (i.e. ADCC) mediated cytotoxicity is relatively unaffected. Other means of effecting target cell death are more resistant. For instance, FasL/Fas interactions and TNF-α induced cytotoxicity remain highly functional under statin treatment (Poggi et al., 2013). In certain circumstances, statin use may even enhance TNF-α production. Another cytokine—IFN-γ—may also exhibit increased secretion from NK cells in response to statins. However, this likely results from interactions with CD14+/CD56+ dendritic-like cells rather than any direct effects on NK cell effector functions (Gruenbacher et al., 2010). Given that the majority of statin treatment experiments use supraphysiologic doses, it remains to be seen how relevant these selectively immunosuppressive effects may be *in vivo*. Lastly, each of the statin experiments cultured NK cells in IL-2. How might IL-12, IL-15, IL-18 or

combination treatments influence the role of mevalonate metabolism in NK cell cytotoxicity?

Now that general principles regarding the immunometabolism of NK cells have been covered, the next section in this thesis will consider the unique metabolic challenges offered by the TME.

INFLUENCE OF THE TUMOR MICROENVIRONMENT ON THE FUNCTION AND METABOLISM OF NK CELLS

For nearly a century, researchers have known that tumors consume far more glucose than non-proliferating tissues (Warburg, Wind, & Negelein, 1927). First described by the German physiologist Otto Warburg, aerobic glycolysis or eponymously "Warburg metabolism" has been identified as a hallmark of cancer in various tumor contexts (Pavlova & Thompson, 2016). In fact, increased glucose consumption in tumors has often been associated with poor prognosis (Almuhaideb, Papathanasiou, & Bomanji, 2011; Som et al., 1980). Glutamine is another principal nutrient critical to the biosynthesis and survival of tumor cells. As such, glutaminolysis proves nearly as important a pathway to cancer metabolism as aerobic glycolysis (Dong, Keibler, & Stephanopoulos, 2017; Pavlova & Thompson, 2016). Whether referring to these pathways or others, the increased metabolic activity of tumor cells produces a microenvironment hostile to anti-tumor cells (e.g. CTLs, NK cells, M1 macrophages, etc.), yet supportive of pro-tumor/immunosuppressive cells (Tregs, MDSCs, etc.) (T. Wang, Liu, & Wang, 2014). Namely, the TME tends to be deprived of nutrients such as glucose, glutamine, and oxygen, yet characterized by an excess of metabolic byproducts such lactate, kynurenine, and protons (Chambers, Lupo, & Matosevic, 2018; Chang et al., 2015; T. Wang et al., 2014). In this section, this thesis will discuss how nutrient restriction, waste product excess, and other soluble factors in the TME potentially antagonize NK cell effector function.

A. Nutrient Restriction

It should come as no surprise that the depletion of certain nutrients common to the metabolism of both tumor and immune cells would inhibit NK cell effector function. Each subsection describes a commonly-depleted nutrient in the TME and evidence suggesting its importance to NK cell function. Note that although this section considers these nutrients individually, *in vivo* these deficiencies present simultaneously with effects that are often synergistic (Loeffler, Heppner, & Juneau, 1991).

1. Glucose Depletion

Rates of glucose consumption often positively correlate with tumor stage or aggressiveness. This is as much a function of glucose uptake as it is the poor vasculature associated with many solid tumors. Because of these factors, intratumoral glucose levels tend to be relatively low (Battista et al., 2016; Busk et al., 2011; Chang et al., 2015; Voelxen et al., 2018).

Few studies directly evaluate the effect of a hypoglycemic environment on NK cell function. The exception comes from Loeffler et al. in the early 1990s, where this group demonstrated that NK cell cytotoxicity against YAC-1 tumors cells was unaffected by hypoglycemia (6 mg/dL) in a murine model with poly (I:C) stimulation. In contrast, less profound hypoglycemia (26 mg/dL) combined with other unfavorable conditions (e.g. hypoxia, acidosis) significantly reduced cytotoxicity (Loeffler et al., 1991). Based on metabolic inhibitor studies mentioned in an earlier section, it appears that the effects of a hypoglycemic microenvironment depend on activation parameters and/or NK cell subsets (Chambers, Lupo, et al., 2018). For instance, IL-12/IL-18 stimulation renders NK

cells impervious to glucose deprivation while IL-12/IL-15 stimulated NK cells rely heavily on glycolytic flux (Keating et al., 2016; Keppel et al., 2015). Illustrating differences between subsets, KIR-educated cells stimulated with membrane-bound IL-21 require aggressive glycolytic inhibition to reduce cytotoxicity while uneducated cells are less tolerant of hypoglycemic environments (Schafer et al., 2019) (see 2. Metabolism of Human NK Cell Effector Functions for more details).

2. Amino Acid Depletion

In the literature, the amino acids most commonly referenced in the context of TME nutrient restriction include glutamine, arginine, and tryptophan (P. J. Murray et al., 2015; O'Neill et al., 2016; Singer et al., 2018). Little is known about intratumoral glutamine, however, several cancer types are known to have high glutamine uptake. Moreover, a commonly upregulated oncogene—*Myc*—induces increased glutaminolysis to support a phenotype of glutamine addiction in some cancers (Gao et al., 2009; Wise et al., 2008). Tryptophan and arginine depletion may be less a direct effect of cancer metabolism and more the result of metabolic activity by MDSCs and other immune cells. Nonetheless, tumors tend to overexpress IDO and arginase-1 which degrade tryptophan (into kynurenine) and arginine, respectively (Singer et al., 2018). While these amino acids have been shown to be critical to the function of T cells and macrophages, their role in NK cells remains to be seen (T. Kobayashi & Mattarollo, 2017; G. K. Lee et al., 2002; Nakaya et al., 2014; O'Neill et al., 2016; Rodriguez, Quiceno, & Ochoa, 2007; Rodriguez et al., 2003; Uyttenhove et al., 2003). At the very least, glutamine availability is important for NK cell cytotoxicity by way of supporting the uptake of other amino acids

to synthesize to cMyc—a transcription factor essential to NK cell effector function (Loftus et al., 2018).

3. Hypoxia

The availability of oxygen in organs depends on the balance between the metabolic demands of constituent cells and the function of their associated microvasculature. *In vivo,* the oxygen concentration in normal tissues may vary widely, from ~1% in bone marrow to 13% in arterial blood (Carreau, El Hafny-Rahbi, Matejuk, Grillon, & Kieda, 2011; Spencer et al., 2014). However, inflammatory or pathological environments may have oxygen tensions nearly ten times lower than corresponding physioxic tissues (Muz, de la Puente, Azab, & Azab, 2015). The tumor contexture comprises one such environment. This can present unique challenges to the NK cell anti-tumor activity (Chambers, Lupo, et al., 2018).

Cell responses to hypoxia tend to follow the effects of the HIF family of transcription factors. Oxygen-dependent hydroxylation promotes the degradation of HIFs, so under conditions of hypoxia HIFs exhibit increased stability and transcriptional activity. The problem of poor cytotoxicity of NK cells in a hypoxic microenvironment is two-fold. First, tumor cells in hypoxic environments resist cell lysis. For example, some tumors cells downregulate the MICA/B (a NKG2D ligand) in response to hypoxia (Schilling, Tetzlaff, Konrad, Li, & Multhoff, 2015; Yamada et al., 2012). However, this MIC shedding could be prevented through exogenous induction of nitric oxide signaling (Barsoum et al., 2011, p. 10; Siemens et al., 2008). In addition, hypoxia can cause autophagy-induced degradation of connexin 43 (important for intercellular connections)

or NK cell effector molecules granzyme B and perforin (Baginska et al., 2013; Tittarelli, Janji, Van Moer, Noman, & Chouaib, 2015; Viry et al., 2014). Second, NK cells prove less effective at killing tumor cells. Overexpression of HIF-1α (as is common in hypoxic environments) downregulates several activating receptors, including the NCRs and NKG2D. Notably, CD16 expression is not affected, leaving ADCC intact (Balsamo et al., 2013). Furthermore, a recent transcriptomic analysis of NK cells under hypoxia up to four days demonstrated that NK cells exhibit reduced ability to secrete cytokines such as IFN-γ, TNF-α, and GM-CSF with IL-15/IL-18 stimulation (Parodi et al., 2018). Just as with glucose deprivation, it is possible that oxygen depletion variably affects NK cell function depending on activating conditions. Whatever conditions support NK cell function despite oligomycin treatment (i.e. OXPHOS inhibition) might very well render NK cells resistant to hypoxic conditions. Of course, this does not consider potential effects of HIF-1α accumulation, which may downregulate mTOR activity in a negative feedback loop during chronic hypoxia (Knaup et al., 2009).

B. Metabolites in Excess

This section will describe the effects of common byproducts of cancer metabolism on NK cell function. Note that these metabolites operate in synergy with the nutrient deprivation alluded to in the previous section.

1. High Lactate and Acidity

To support aerobic glycolysis, tumors cells reduce pyruvate to lactate, which regenerates NAD+ from NADH. High rates of glycolysis lead to accumulating levels of

lactate and protons, as the latter are co-transported via monocarboxylate transporters (MCT1/4) (Harmon et al., 2018; Pavlova & Thompson, 2016). Because of this co-transport, it can be rather challenging to separate the effects of lactate from the changes in extracellular pH *in vivo.* However, *in vitro* studies reveal that low pH and exogenous lactate can both significantly reduce NK cytolytic activity (B. Fischer, Müller, Fisch, & Kreutz, 2000; B. Fischer, Müller, Fischer, Baur, & Kreutz, 2000; Harmon et al., 2018; Husain, Huang, Seth, & Sukhatme, 2013; Loeffler et al., 1991; Severin et al., 1994). There may be several mechanisms mediating this immunosuppression. In the context of colorectal liver metastasis, low pH of the tumor milieu was associated with decreased mitochondrial mass, increased ROS, and more frequent apoptosis in liver-resident NK cells. This effect was more pronounced in $CD56^{bright}$ NK cells and may explain the poor infiltration of NK cells in this tumor model. Moreover, the lactic acid gradient inversely correlated with the liver-resident NK cell population. Notably, exogenous addition of an ROS scavenger could partially rescue this dysfunctional phenotype (Harmon et al., 2018).

Brand et al. discovered that LDHA expression and lactate levels were directly correlated, with high lactate potentially inhibiting nuclear translocation of nuclear factor of activated T cells (NFAT) in NK cells (and T cells) leading to reduced IFN-γ production. The authors hypothesize that high extracellular lactate inhibits lactic acid secretion from NK cells, thereby reducing intracellular pH (Brand et al., 2016). Supporting this, low pH is known to suppress calcineurin, a phosphatase that regulates NFAT (Hisamitsu, Nakamura, & Wakabayashi, 2012). Furthermore, decreased expression of LDHA results in slower tumor growth in murine models of melanoma and

breast cancer (Brand et al., 2016; Serganova et al., 2018). The former model corresponded with increased T/NK cell infiltration and IFN-γ production, while the latter model corresponded with decreased HIF-1α signaling, impaired vascularization, and reduced infiltration of tumor-promoting tumor-associated macrophages (TAMs) (Brand et al., 2016; Serganova et al., 2018).

Lactate treatment has also been shown to reduce expression of activating receptor NKp46, granzyme B, and perforins in NK cells. Remarkably, that same study showed that a ketogenic diet improved anti-tumor immune activity and reduced numbers of MDSCs in a murine model of pancreatic cancer, presumably by decreasing glucose and therefore the substrate for lactate production (Husain et al., 2013).

2. Kynurenine

Recall that in the TME, tryptophan tends to be depleted as its degradation product—kynurenine—accumulates (Singer et al., 2018). In CD8+ T cells, kynurenine excess hampers proliferation and suppresses effector function while inducing differentiation of tumor-promoting Tregs—likely through the aryl hydrocarbon receptor (AhR, a transcription factor). In several contexts, kynurenine is thought to contribute to an immunosuppressive environment (N. T. Nguyen et al., 2010; Opitz et al., 2011; Platten, Wick, & Eynde, 2012). However, with regard to NK cells the situation is less clear. Early studies essentially agree with findings for T cells. Namely, kynurenine (and other tryptophan catabolites) suppress NK cell proliferation and decrease expression of activating receptors NKp46 and NKG2D (Della Chiesa et al., 2006; Frumento et al., 2002). More recent evidence paints a different picture though. For example, a research

group showed in 2013 that AhR activation (for which kynurenine is an agonist) potentiates both IFN-γ production and cytolytic activity in NK cells (Shin et al., 2013). This is especially true of the $CD56^{bright}$ NK cell subset, which displays greater expression of AhR and upregulates this receptor in response to cytokine activation (though as a general trend, AhR expression decreases with maturation) (Moreno-Nieves, Mundy, Shin, Tam, & Sunwoo, 2018). In light of this and other studies, kynurenine excess may very well increase surface expression of NKp44 and CD25 as well as enhance secretion of pro-inflammatory cytokines such as IFN-γ, TNF-α, and GM-CSF (Moreno-Nieves et al., 2018; Opitz et al., 2011; Shin et al., 2013). On the other hand, AhR activation also promotes NK cell secretion of the anti-inflammatory cytokine IL-10 (Wagage et al., 2014). Moreover, the induction of CD25 (high affinity receptor subunit for IL-2) in NK cells may limit the availability of IL-2 in the TME in a manner similar to Tregs (Moreno-Nieves et al., 2018). Needless to say, more investigation is needed to clarify the effect of kynurenine on the anti-tumor activity of NK cells. It may be possible kynurenine exhibits pleiotropic effects on NK cell function divergent from its role as an AhR agonist.

3. Adenosine

A product of ATP degradation, adenosine is a purine nucleoside whose extracellular levels in tumors can be up to 100-fold greater than those found in normal tissues (Ohta et al., 2006). Two ectonucleotidases—CD39 and CD73—catalyze the formation of adenosine in response to certain signals, the most important of which may be hypoxia (Allard, Beavis, Darcy, & Stagg, 2016; Sitkovsky et al., 2014). Extracellular adenosine can initiate signaling in immune cells through any one of four G-protein

coupled protein receptors (GPCRs). These include the receptors A1, A2A, A2B, and A3 (J. Wang & Matosevic, 2018). A1 and A3 signaling may actually increase NK cell cytotoxicity, while A2A and A2B are associated with immunosuppression (Chambers, Lupo, et al., 2018). Presumably, the pro-inflammatory or immunosuppressive effects of adenosine are mediated by changes in cAMP (cyclic AMP). A1/A3 inhibit adenylate cyclase (decreasing cAMP levels) while A2A/A2B activate adenylate cyclase (increasing cAMP levels) (see Figure 17) (Harish, Hohana, Fishman, Arnon, & Bar-Yehuda, 2003; Priebe, Platsoucas, & Nelson, 1990; Raskovalova et al., 2005; J. Wang & Matosevic, 2018). Adenosine can also be transported across the cell membrane by adenosine transporters, after which the action of adenosine kinase regenerates AMP. This activates AMPK associated pathways which promote catabolic metabolism and suppress anabolism (partially through mTORC1 inhibition) (Aymerich, Foufelle, Ferré, Casado, & Pastor-Anglada, 2006; Herzig & Shaw, 2018; Thorn & Jarvis, 1996; Villanueva-Paz et al., 2016).

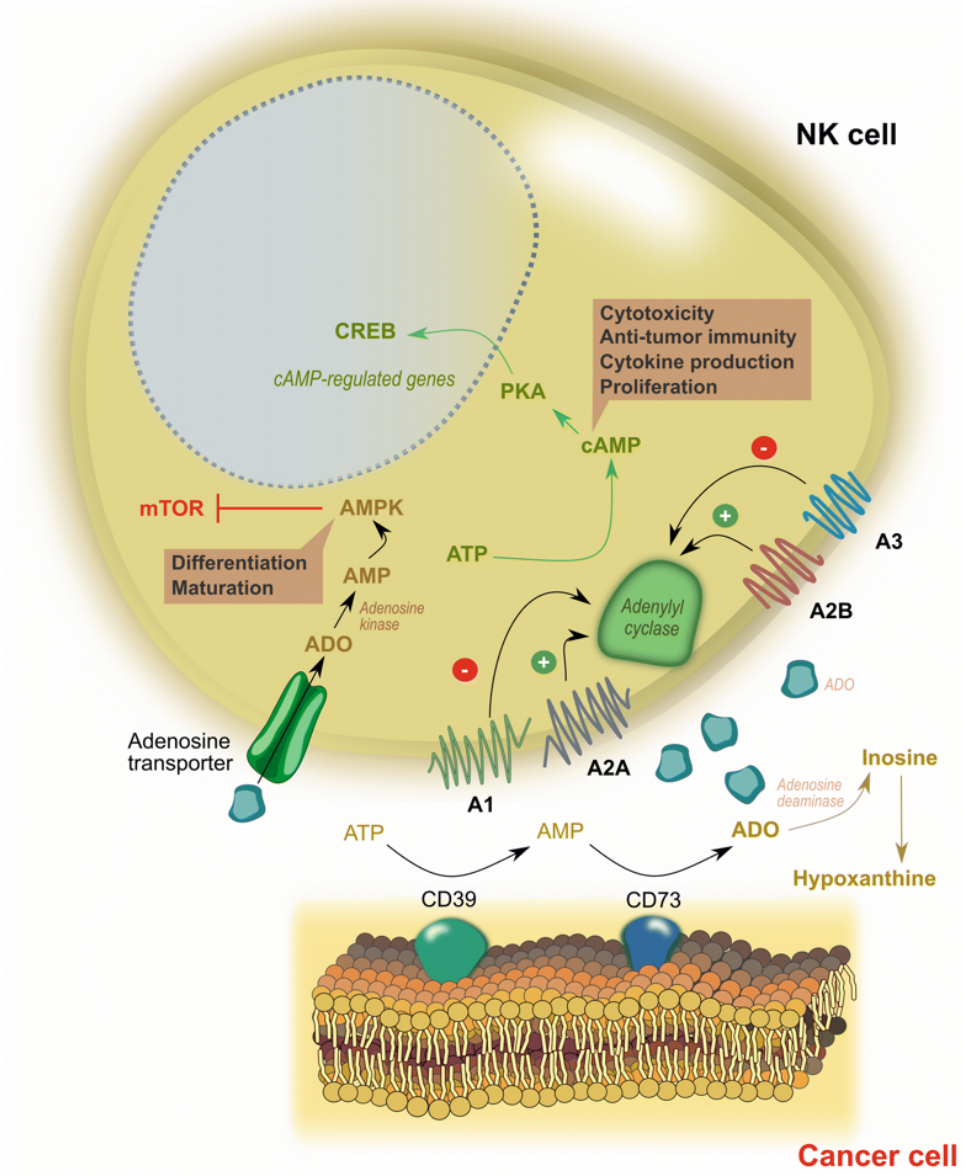

Figure 17. Adenosinergic Signaling in NK Cells. CD39 dephosphorylates ATP in AMP, while CD73 generates extracellular adenosine (ADO) from AMP. This adenosine can signal through one of four GPCRs that regulate intracellular cAMP levels and thereby affect NK cell effector functions in a cAMP-dependent fashion. Extracellular adenosine can also be transported across the cell membrane where it is phosphorylated by adenosine kinase to produce AMP. This leads to activation of AMPK-associated pathways which regulate cell metabolism. A third fate for adenosine is degradation to inosine by adenosine deaminase. Note: PKA = Protein Kinase A; CREB = cAMP responsive element binding protein. Adapted from (J. Wang & Matosevic, 2018).

Of the adenosine GPCRs, A2A is the most highly expressed in NK cells (Young et al., 2018). Moreover, chemical and genetic inhibition of the A2A receptor has been shown to enhance tumor control and delay tumor progression. The mechanism has yet to be fully elucidated, but evidence suggests that adenosine signaling through A2A suppresses NK cell maturation. Consequently, A2A inhibition results in a greater proportion of $CD56^{dim}$ NK cells, which tend to be more cytotoxic (Young et al., 2018).

At least one study demonstrated that adenosinergic signaling in NK cells may vary according to the cytokine stimulation regimen. In 2018, Chambers and colleagues compared the effects of adenosine signaling on NK cell effector function after 24 hours of exposure to IL-2 alone, IL-12 + IL-15, or IL-15 alone. In the last two groups, adenosine significantly increased IFN-γ secretion, especially in the $CD56^{bright}$ NK cell subset. These same groups also exhibited decreases in NKG2D expression with adenosine exposure (Chambers, Wang, et al., 2018). Given that an mTOR inhibitor (Torin-1) negated most of adenosine's effects on IFN-γ secretion, mTOR may mediate some of adenosine's effects on NK cells. Surprisingly, adenosine treatment minimally affected NK cell cytotoxicity against lung tumor cell A549 *in vitro*. However, subsequent treatment with an adenosine deaminase inhibitor (prevents degradation of extracellular adenosine) did significantly decrease *in vitro* NK cell cytotoxicity (Chambers, Wang, et al., 2018). This may be more representative of the chronically high adenosine concentrations present in the TME.

Lastly, adenosine treatment also reduces baseline glycolysis and OXPHOS, as well as the respective maximal capacities of these metabolic pathways. This regulation is likely at the transcriptional level as adenosine treatment is associated with downregulation of gene transcripts for the rate-limiting glycolytic enzymes LDHA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Chambers, Wang, et al., 2018). Note that although the direct effects of adenosine on NK cell effector functions are becoming more clear, indirect effects through adenosine's influence on other immune cells (especially immunosuppressive subsets like MDSCs and Tregs) may be the most relevant to tumor growth and survival (Young, Mittal, Stagg, & Smyth, 2014).

4. Oxysterols and Cholesterol

Recall that SREBP induces the upregulation of the citrate-malate transporter and that its inhibition correlates with decreased NK cell cytotoxicity (Assmann et al., 2017). Cholesterol and oxysterols (oxidized derivatives of cholesterol such as 25-HC or 27-HC) can inhibit SREBP and could therefore potentially affect NK cell effector function (Adams et al., 2004; Li, Long, Huang, Chen, & Xia, 2018; Shimano & Sato, 2017). A number of cancers exhibit increased levels of cholesterol or oxysterols, which are associated with decreased patient survival in such diverse cancer types as breast cancer, glioblastoma, melanoma, and colon cancer (Eibinger et al., 2013; Javitt, 2015; Kuzu, Noory, & Robertson, 2016; Nelson, 2018; Rossin et al., 2019). While treatment with 25-HC has been shown to directly inhibit NK cell effector function, the effects of increased cholesterol are less clear. In fact, cholesterol levels may positively correlate with NK cell effector function (Assmann et al., 2017; Hillyard et al., 2007; Raemer et al., 2009). It is

likely that cholesterol levels affect a number of different processes besides the citrate-malate transporter, each of which may have competing effects on NK cell cytotoxicity. Inferring the effects of cholesterol in the tumor milieu may very well depend on how much cholesterol is converted to oxysterols by sterol hydroxylases. Glioblastoma has been shown to overexpress cholesterol 25-hydroxylase while macrophages are known to upregulate cholesterol 25-hydroxylase in response to certain stimuli (Diczfalusy et al., 2009; Eibinger et al., 2013; Park & Scott, 2010). Moreover, macrophages generally express high levels of sterol 27-hydroxylase (Babiker et al., 1997). Much like the other metabolites, for oxysterols/cholesterol the role of other immune cells of the tumor contexture in NK cell metabolism and function should not be underestimated.

C. Other Soluble Factors

Numerous other soluble factors present in the TME may potentially affect NK cell metabolism, yet—strictly speaking—are neither nutrients nor metabolites. Such factors include pro-inflammatory cytokines (e.g. IL-12, IL-15, and IL-2), immunosuppressive cytokines (e.g. IL-6, IL-10, and TGF-β) as well as prostaglandin E2 (PGE2) and soluble NCR ligands (which induce the downregulation of activating receptors) (see Figure 18) (Hasmim et al., 2015) Activating cytokines have already been extensively covered, so this section will consider two soluble factors with some of the most important effects on NK cell metabolism and function—TGF-β and PGE2.

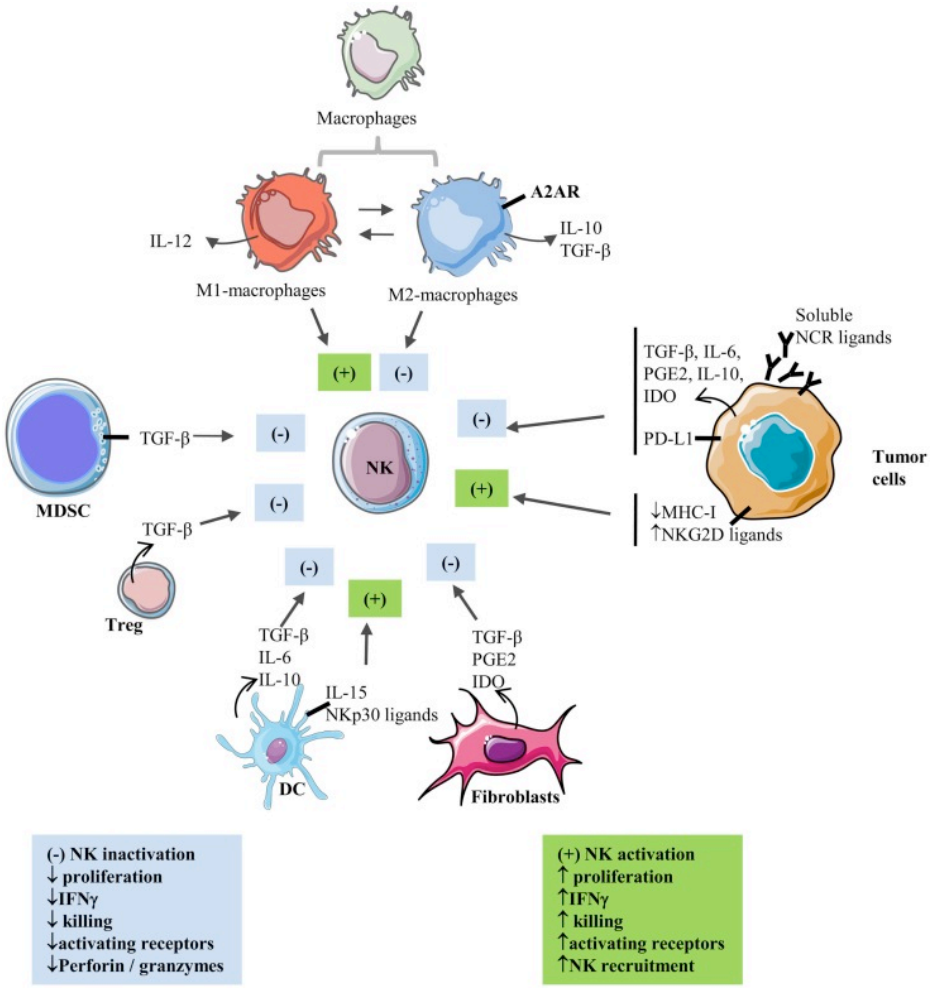

Figure 18. Soluble Factors in the TME. In addition to tumor cells, a variety of immune cells contribute soluble factors to the TME which either act to inhibit (-) or enhance (+) NK cell activation and effector function. Adapted from (Hasmim et al., 2015).

1. TGF-β

A cytokine belonging to the bone morphogenetic protein (BMP) family, TGF-β exhibits pleiotropic effects. Virtually, all immune cells express TGF-β receptors while most cells secrete TGF-β (of which there are three homologues) (Neuzillet et al., 2015; Viel et al., 2016). Moreover, the effects of TGF-β are primarily immunosuppressive, as it inhibits the antitumor activity of NK cells and CTLs (Flavell, Sanjabi, Wrzesinski, & Licona-Limón, 2010; Massagué, 2008, p.). A variety of cancers exhibit progressively elevated levels of TGF-β as the malignancy advances (Ivanović et al., 2003; Massagué, 2008; Neuzillet et al., 2015). In recent years, researchers have revealed several potential mechanisms behind the immunosuppressive action of TGF-β on NK cells.

In 2014, Donatelli and colleagues observed that TGF-β treatment upregulated the expression of miR-183 in a murine model of NK cells. This miR-183 expression then inhibited expression of the signaling adaptor protein DAP12. Given that DAP12 associates with the activating receptor NKp44, surface expression of NKp44 was also reduced (despite no change in cytoplasmic expression of NKp44) (Donatelli et al., 2014). Two years later, Viel et al. presented evidence that TGF-β affected cytokine-activated (high-dose IL-15) NK cell effector function and metabolism by disabling mTOR signaling. Investigations showed that the effects of TGF-β or rapamycin (mTORC1 inhibitor) treatment on NK cells were incredibly similar. Specifically, both treatments reduced NK cell metabolic capacity (i.e. OXPHOS and glycolytic capacity) and impaired cytotoxicity against YAC-1 target cells (Viel et al., 2016). Even more, constitutive TGF-

β signaling and mTOR knockout induced similar dysfunctional phenotypes in a murine model (Viel et al., 2016). The study also showed murine NK cells with TGF-β receptor knockout displayed greater granzyme B and nutrient receptor (i.e. CD71, CD98) expression compared to wild type *in vitro* and improved control of lung metastasis *in vivo* (Viel et al., 2016).

On the other hand, a 2018 study in human NK cells found little evidence of impaired mTOR signaling due to TGF-β treatment—at least in the short term (~18 hours). TGF-β treatment only affected mTOR signaling after five days of cytokine activation (IL-2 or IL-12/IL-15) (Zaiatz-Bittencourt, Finlay, & Gardiner, 2018). Furthermore, the authors found that TGF-β treatment induced metabolic dysfunction in NK cells similar to that witnessed by Viel et al (Viel et al., 2016; Zaiatz-Bittencourt et al., 2018). This finding suggests that TGF-β can inhibit NK cell metabolism independent of mTOR signaling. Other differences depend on CD56 expression. While TGF-β decreased IFN-γ synthesis in both $CD56^{bright}$ and $CD56^{dim}$ NK cells, only the former subset displayed reductions in granzyme B and TRAIL expression. Alas, the inconsistencies between the most recent studies on NK cell function and TGF-β might be a result of interspecies differences (mice vs. men) or the cytokine activation regimen (i.e. long-term vs. short-term, IL-2 or IL-12/IL-15 vs IL-15 only) (Viel et al., 2016; Zaiatz-Bittencourt et al., 2018).

In a murine model of *Kras*-driven lung cancer, Cong et al. tie TGF-β exposure of NK cells to the upregulation of fructose-bisphosphatase 1 (FBP1). FBP1 is a gluconeogenic enzyme that directly inhibits glycolysis. In their model, Cong et al.

categorize NK cells into stages 1, 2, or 3, which correspond to tumor initiation, promotion, and progression, respectively. With higher stages (i.e. tumor progression), NK cells displayed gradual reductions in granzyme B, perforin, CD107a (degranulation marker), IFN-γ, and TNF-α expression. In addition, activating receptor NKG2D became less prevalent while inhibitory receptor NKG2A became more prevalent. Furthermore, by stage 3, NK cells expressed *Fbp1* at levels 69-fold higher than wild type NK cells (Figure 19) (Cong et al., 2018; Isaacson & Mandelboim, 2018).

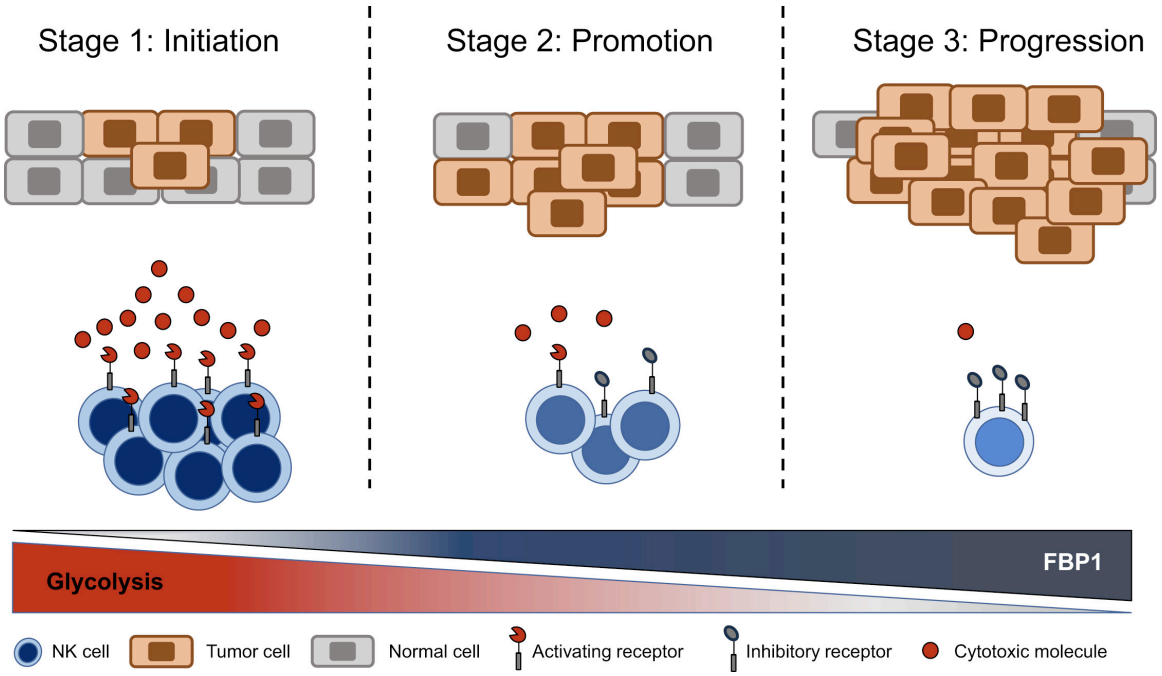

Figure 19. Increasing FBP1 Expression Leads to Dysfunctional NK Cells during Lung Tumor Progression. In a murine KRAS-driven lung cancer, as the tumor advances NK cells become progressively less cytotoxic. Dysfunction emerges from increasing FBP1 expression, which impairs metabolic fitness by inhibiting glycolysis. Adapted from (Isaacson & Mandelboim, 2018).

Stage 3 NK cells have an irreversible state of dysfunction characterized by poor cytotoxicity, impaired viability, and reduced glycolysis. Stage 2 NK cells have a mild form of dysfunction, but this can be partially recovered with a FBP1 inhibitor. In fact, adoptively transferred NK cells co-delivered with a FBP1 inhibitor slowed tumor growth in a murine model. Notably, inhibition of glycolysis (by 2DG administration) in concert with FBP1 inhibition recovered NK cell cytotoxicity but not viability. This suggests that the effects of FBP1 on NK cell effector function may depend on more than metabolism (Cong et al., 2018).

As shown above, the immunosuppressive effects of TGF-β on NK cells may be mediated by a variety of downstream effectors, depending on activation conditions. Experiments have predominately considered TGF-β in isolation, so it remains to been how TGF-β might synergize with other immunosuppressive factors in the TME to suppress NK cell function and metabolism.

2. PGE2

Prostaglandins such as PGE2 are synthesized from arachidonic acid through the action of cyclooxygenases (constitutively active COX-1 and inducible COX-2). PGE2 is produced primarily by myeloid and stromal cells. (Kalinski, 2012). However, PGE2 production can be significant in cancer cells under certain conditions. For example, colorectal adenoma and carcinoma cells increase PGE2 in hypoxic environments (as might be found in the TME). This is mediated through HIF-1α, which upregulates COX-2 expression (Kaidi, Qualtrough, Williams, & Paraskeva, 2006). Increased PGE2 can in

turn inhibit NK cell IFN-γ synthesis, resulting in decreased NK cell cytotoxicity (Bankhurst, 1982; Goto, Herberman, Maluish, & Strong, 1983; Joshi, Zhou, Cuchens, & Jones, 2001; Walker & Rotondo, 2004). The mechanism for decreased IFN-γ synthesis is not well-understood, but for IL-15 activated NK cells it may be related to the PGE2-induced reduction of γ_c expression (Joshi et al., 2001). Meanwhile, in IL-12/IL-18 stimulation, PGE2 suppression of IFN-γ production is associated with increased cAMP levels, much like adenosine signaling mentioned previously (Walker & Rotondo, 2004; J. Wang & Matosevic, 2018). PGE2 may also impair NK cell cytotoxicity indirectly by inducing immunosuppressive cell types, such as Tregs, M2 macrophages, and MDSCs (Heusinkveld et al., 2011; A. C. Ochoa, Zea, Hernandez, & Rodriguez, 2007; Sinha, Clements, Bunt, Albelda, & Ostrand-Rosenberg, 2007; Whiteside & Jackson, 2013).

DISCUSSION

Against hematological cancers, NK cell-based immunotherapy has become an increasingly important treatment modality. However, results in solid cancers are less promising (Cheng, Chen, Xiao, Sun, & Tian, 2013; Fang et al., 2017). That lack of success can be understood partially through the lens of immunometabolism (O'Brien & Finlay, 2019). As shown above, the high metabolic activity of tumors (which involves substantial rates of glucose and glutamine consumption) produces a metabolically inhospitable environment that significantly suppresses NK cell anti-tumor activity (Chang et al., 2015; Dunphy et al., 2018; Hirayama et al., 2009; Ho et al., 2015; B. P. Lieberman et al., 2011; Still & Yuneva, 2017; Urasaki, Heath, & Xu, 2012; L. Zhu, Ploessl, Zhou, Mankoff, & Kung, 2017). To counter immunometabolic suppression, two broad strategies come to mind: researchers can either render NK cells insensitive to metabolic challenges in the TME or make the TME more hospitable. Regardless of the strategy employed, both require an understanding of the fundamental relationship between NK cell metabolism and NK cell function. Central to that understanding is the notion that that relationship is neither static nor immutable, but dynamic and fluid.

1. Reducing NK Cell Vulnerability

Ostensibly, many studies on NK cell metabolism present inconsistent results, but it is essential that one consider the unique metabolic dependencies of different activation regimens for different NK cell subpopulations. For instance, IL-12 + IL-18 activates murine NK cells in such a way that glucose deprivation does not affect IFN-γ production.

(Keppel et al., 2015). Similar "glucose independence" of cytotoxic functions can be induced by long-term (14-21 days) exposure of KIR-educated human NK cells to membrane-bound IL-21 or overnight, low-dose IL-15 stimulation of NKG2A-educated human NK cells (Pfeifer et al., 2018; Schafer et al., 2019). On the other hand, the receptor-mediated (i.e. NK1.1, Ly49D) and IL-12/IL-15 mediated IFN-γ production of murine NK cells remains heavily metabolism-dependent (both glycolysis and OXPHOS) (Keppel et al., 2015). By carefully considering activation conditions, researchers can strategically expand robust NK cells and optimize adoptive NK cell therapies for solid malignancy.

Cytokine activation, however, cannot fully protect against the immunometabolic suppression of the TME. In fact, despite their glucose independence, IL-12/IL-18 activated NK cells remain vulnerable to suppressive adenosinergic signaling (Beavis et al., 2013; Keppel et al., 2015). In combination with appropriate cytokine activation regimens, researchers could design A2A receptor knockout NK cells, conduct A2A receptor blockade, or administer antibodies against the ectonucleotidases CD39 and CD73. Each of these three therapies has been shown to increase NK anti-tumor activity (Beavis et al., 2013; Chambers, Lupo, et al., 2018; Hatfield & Sitkovsky, 2016; J. Wang, Lupo, Chambers, & Matosevic, 2018). Moreover, the last method has been shown to enhance NK cell cytotoxicity via ADCC as well as through inhibition of adenosine metabolism (Häusler et al., 2014). Another method to reduce NK cell vulnerability would be glutaminase inhibition. Because NK cell do not require glutamine anaplerosis (only availability), a glutaminase inhibitor can suppress tumor glutaminolysis to maintain the

glutamine pool (Loftus et al., 2018). Of course, one must be wary of the potential deleterious effects on other anti-tumor immune cells. For example, blocking glutamine metabolism may also inhibit T cell activation, proliferation and function (Carr et al., 2010; R. Wang et al., 2011). In this case, a safer solution might be GSK3 or proteasome inhibition, which could increase cMyc expression in NK cells despite a glutamine-poor environment (Loftus et al., 2018). Though NK cell metabolism was not explicitly considered, multiple studies have demonstrated increased NK cell anti-tumor activity in response to GSK3 inhibitors (Cichocki et al., 2017; Parameswaran et al., 2016). A number of other inhibitors also improve NK cell antitumor activity, such as ones against FBP1, TGF-β receptor I/II, and miR-183 (Cong et al., 2018; Donatelli et al., 2014; Neuzillet et al., 2015; Zaiatz-Bittencourt et al., 2018).

2. Promoting a Less Hostile TME

Making the TME more hospitable can be via direct methods (on the environment) or indirect methods (on cancer cells or other immunosuppressive cell types). Of course, not all methods can be so neatly delineated. As an example, systemic administration of sodium bicarbonate can reverse NK cell dysfunction secondary to local acidosis and improve NK cell-dependent control of lymphoma (Pötzl et al., 2017). Another potential method to reverse acidosis is proton pump inhibitor (PPI) administration, which has been shown to exhibit potent anti-tumor effects (Huber et al., 2017). These effects result not only from a direct impact on cancer cells, but also from enhanced immunity (Huber et al., 2017; Milito et al., 2010). Even more, in a model of colorectal liver metastasis, the

apoptosis-inducing effects of lactate-mediated acidification were surprisingly countered by limiting glucose levels through dieting (i.e. ketogenic diet). Presumably, the limited glucose reduces the substrate available for LDHA (Harmon et al., 2018).

Supplemental oxygen has also shown utility in countering the immunosuppressive effects of the TME. Oxygen delivery can be achieved by various techniques, but the most encouraging outcomes result from respiratory hyperoxia, especially with hyperbaric oxygen (Graham & Unger, 2018). In fact in mice, sixty percent respiratory oxygenation reduces intratumoral hypoxia, which mitigates the insidious effects of HIF-1α on MHC class I/MICA/B expression, decreases the prevalence of immunosuppressive adenosine, discourages MDSC proliferation (likely changing the cytokine milieu), and decreases PD-L1 expression (Hatfield et al., 2015; Leone, Horton, & Powell, 2015; Qian et al., 2019; Schilling et al., 2015; Yamada et al., 2012). Notably, this systemic oxygenation has been shown to inhibit the growth of B16.F10 melanoma tumors in a T cell and NK cell-dependent fashion (Hatfield et al., 2015). Hyperoxygenation also enhances the anti-tumor efficacy of more traditional therapies such as chemotherapy and radiotherapy (Cade & McEwen, 1978; Mast & Kuppusamy, 2018; Watson et al., 1978).

CONCLUSION AND FUTURE DIRECTIONS

There is a timeless adage: "you are what you eat." It turns out that NK cells are no exception. NK cell metabolism and NK cell function possess an intimate relationship. Indeed, this is true of every cell. To cell function, metabolism is both master and servant. Historically, this has been a liability in the immune system's constant fight against malignancy. However, new progress in immunometabolism may allow researchers to change the players—and the game. Until recently, researchers really understood only one of the key players—the T cell—but gaps in the knowledge of NK cell metabolism are quickly closing. The future of immunotherapy promises the development of NK cells (and T cells) that can flourish in the metabolically restrictive TME to better eradicate solid and hematological cancers.

And yet, much work remains. Knowledge of cytokine and receptor-mediated NK cell activation and inhibition—especially inhibition—in humans remains sparse (O'Brien & Finlay, 2019). Furthermore, future studies need to at least optimize timing, quantity, and delivery in activation schemes to render NK cells more metabolism-independent and/or cytotoxic. Studies should also address the influence of inorganic ions such as sodium, potassium, and calcium on NK cell anti-tumor activity. How these ions are regulated in the TME could have major impacts on NK cell cytotoxicity (Singer et al., 2018). For instance, the extracellular calcium concentration can determine how immunogenic NK cell-mediated tumor cell death may be. Higher calcium concentrations encourage necrosis, while lower calcium concentrations promote less immunogenic, apoptotic pathways (Backes et al., 2018). Meanwhile, the necrotic core of most tumors

may induce local hyperkalemia that impairs ion transport, which can have deleterious effects on AKT-mTOR signaling (Eil et al., 2016). This leads to another point. While *in vivo* experiments are clearly in need, less cumbersome *in vitro* studies could be significantly improved by exposing cell targets to the same conditions as NK cells in cytotoxicity assays. Virtually all studies on immunometabolism and NK cell effector function expose NK cells to a hostile environment (e.g. glucose-free media, hypoxia, etc.) only to restore favorable conditions during cytotoxicity assays. However, clearly there are interaction effects mediated by environmental conditions (e.g. high calcium concentration) that could not be observed otherwise.

Discovering the molecular mechanisms of NK cell metabolic independence or dependence should also be a priority. Put another way, how and why do different metabolic pathways affect NK cell cytotoxicity? Is it at the transcriptional level? Translational? Proteomic? Moreover, which of the four stages in NK cell granule exocytosis requires a metabolic signal (Topham & Hewitt, 2009)? For instance, while the effects of glycolysis on IFN-gamma synthesis in T cells can be explained through transcription regulation mediated by the glycolytic enzyme GAPDH, current evidence supports no such mechanism in NK cells. (Chang et al., 2013; Donnelly et al., 2014).

Considering all the aforementioned points, how do these processes vary among different NK cell subsets? Previous research has segregated NK cells according to CD56 expression or “education” status, but studies have not investigated the interaction effects of such designations (Keppel et al., 2015; Pfeifer et al., 2018; Schafer et al., 2019). Furthermore, with regard to “education” status there are differences in metabolism

between KIR and NKG2A-educated NK cells that should be clarified (Pfeifer et al., 2018; Schafer et al., 2019). In addition, tissue-resident cells exhibit a nutrient receptor profile different from that of circulating NK cells (Salzberger et al., 2018). It would stand to reason that the metabolism might also vary. Given trNK cells generally have more amino acid transporters (and less glucose transporters) than cNK cells, does this mean trNK cells rely less on glycolytic metabolism? How does this affect anti-tumor activity? Even before answering these questions, researchers need to establish the relative importance of trNK cells vs. cNK cells in immunosurveillance for different cancer types.

Finally, the impact of systemic metabolism in chronic disease on NK cell effector function needs to be more closely examined. It is well-known that obesity, cardiovascular disease (CVD), and diabetes are associated with an increased incidence of cancer (Calle & Kaaks, 2004; Hasin et al., 2013; Michelet et al., 2018; Ohkuma, Peters, & Woodward, 2018; Renehan, Tyson, Egger, Heller, & Zwahlen, 2008; Suzuki et al., 2017). It is likely that the altered systemic metabolism that characterizes these chronic conditions may lead to impaired cancer immunosurveillance by NK cells (Jung et al., 2018; J. H. Kim et al., 2019; Michelet et al., 2018; Spielmann et al., 2017; Tobin et al., 2017). In fact, one recent paper explicitly described a mechanism whereby obesity induced increased peroxisome proliferator-activated receptor (PPAR) activity, which promoted dysregulated NK cell metabolism (by enhancing FAO) and poor granule polarization. The effects of diabetes, CVD, and other chronic illnesses (especially those with a metabolic component) on NK cell metabolism should be explored with the same rigor. This is particularly important in light of the complex effects on NK cell metabolism and function that metformin and

statins (standard treatments for diabetes and CVD, respectively) may elicit (Chimento et al., 2019; Hillyard et al., 2007; Poznanski et al., 2018; Raemer et al., 2009; Toru Tanaka et al., 2007).

This thesis has provided foundations on NK cell biology, immunometabolism and how these two phenomena meet in the TME. It is hoped that this work has both informed and inspired the next generation of immunometabolic researchers. NK cell-based immunotherapy has seen many challenges, but the triumphs are soon to come.

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